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STUDIES ON OSMOTIC EQUILIBRIUM AND ON THE KINETICS OF OSMOSIS IN LIVING CELLS BY A DIFFRACTION METHOD*

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For the study of osmotic phenomena in living cells, and of their permeability to water and to solutes, the eggs of the sea urchin, *Arbacia punctulata*, have proved to be excellent material (1) The cells are spherical and of very uniform size, their spherical shape is maintained for hours in anisotonic solutions in which the course of volume change may be followed from the beginning to equilibrium by measuring cell diameter under the microscope However, such direct measurements, while accurate, are laborious, and they suffer from the disadvantage that they are based upon individual cells which are subject to a certain degree of variability It seemed desirable therefore to search for a method of measurement by means of which the variability of individual cells would be averaged out An adaptation of a diffraction method for measuring small objects, first described by Thomas Young (2) in 1813 was found suitable for our purposes By this method we were able to measure with satisfactory accuracy the average diameter of large numbers of cells Moreover since measurements can be made rapidly and at short intervals of time, the course of swelling or shrinking of cells may be followed from beginning to completion There is the further advantage ~~that~~ that the cells can be measured while in suspension and ~~continuously~~ stirred

In the course of testing the method we have performed many measurements of *Arbacia* eggs in osmotic equilibrium and during the course

* Aided by grants from the Ella Sachs Plotz Foundation and the Faculty Research Committee of the University of Pennsylvania

of swelling or shrinking in anisotonic solutions, and have obtained data which considerably extend our previous direct measurements. Thus, we have restudied the applicability of the Boyle-van't Hoff law to these cells and obtained more extensive data on the amount of the osmotically inactive material which corrects total cell volume to solvent volume. Second, we have reinvestigated the kinetics of osmotic volume change and thereby gained further information on the permeability of the cell to water. Third, rate of penetration of a solute (ethylene glycol) has been measured.

The results of these experiments and a description of the diffraction method are reported in this paper.

Principle of Method

When a beam of light from a point source is passed through a suspension of objects, appropriate in size and shape, diffraction of the light occurs, causing the appearance of a series of concentric bright rings around the image of the original light source. The size of these rings depends upon the size of the objects, hence the diffraction phenomenon may be used as a method of measurement. Since each individual object contributes to the diffracted light, a single measurement of the diffraction pattern gives the average size of all of the objects in the suspension. This principle was first applied by Thomas Young (2) in his measurements of a great variety of biological objects such as erythrocytes of different animals, pus cells, vegetable cells, wool fibres, and so forth. In more recent years the method has especially been used to measure the diameter of mammalian erythrocytes by Ponder (3), Emmons (4), Pijper (5), Millar (6), and others. These latter studies, and particularly the refinements introduced by Ponder (3), have led us to apply the diffraction method to measurement of much larger cells, the unfertilized eggs of the sea urchin.

Method

A satisfactory diffraction pattern is obtained by illuminating a suspension of sea urchin eggs by parallel monochromatic light from a slit source. As viewed through a telescope this pattern consists of alternate dark and light bands (minima and maxima of intensity) symmetrically arranged on either side of the image of the slit. The angular displacement of a given diffraction band from the central image of the slit is determined by setting the cross-hairs of the telescope upon it and reading on a scale the angle through which the telescope has been rotated. From such measurements the average diameter of the objects causing diffraction may be computed.

Millar (6) has shown that the positions of maximum and minimum intensity of light within such a diffraction pattern are given by the equation

$$d \sin \theta = \frac{s}{\pi} \lambda \quad (I)$$

where d is the diameter of the objects causing diffraction, λ the wave length of light, θ the angular displacement from the central image of the particular minimum or maximum under observation, and s a constant characteristic of the particular maximum or minimum under observation. A table of theoretical values of $\frac{s}{\pi}$ may be found in Millar's paper (6). Owing to the relatively large size of *Arbacia* eggs (approximately 75μ in diameter) the angles of diffraction are sufficiently small so that for $\sin \theta$ one may substitute $\tan \theta$, which in the present instrument is proportional to the scale deflection, s . Equation (I) therefore becomes

$$d s = C \quad (II)$$

where C is a calibration factor characteristic of each maximum and minimum

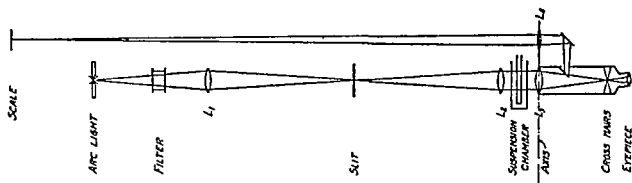


FIG 1 Diagram of diffraction apparatus

The details of the apparatus are shown in Fig 1. Light from a concentrated mercury arc (similar to that described by Allen and Ponder (7)) passes through a liquid filter¹ which absorbs practically all of the light in the mercury spectrum excepting the green line (λ 5461), an image of the arc is formed by a lens, L_1 , on a vertical slit. By means of the collimating lens L_2 the light is made parallel as it passes through the suspension of the cells. The telescope lens, L_3 , forms an image of the slit in the plane of the cross hairs which are in the form of an elongated λ , and which are viewed through an ocular. Precautions must be taken to reduce as far as possible stray light in the optical system.²

The telescope is rotated about a vertical axis by means of a screw-drive. The image of a horizontal scale, by means of which the angle of rotation is measured

¹ This filter contains potassium bichromate and a neodymium salt (8)

This was accomplished by introducing suitable diaphragms to eliminate scattered light, and, to avoid troublesome reflections from the optical surfaces the collimating lens and the chamber containing the cell suspensions were set slightly askew

is projected onto the plane of the cross-hairs by an auxiliary telescope lens, L_4 , and appropriate mirrors. A footswitch serves to turn on the lamp which illuminates the scale and at the same time closes a shutter at the slit. This enables the observer, after setting the cross-hairs on a diffraction band, to read the deflection of the telescope on the scale without moving his eye.

The chamber in which the cells are suspended is made of plate glass, it has an internal thickness of 5 mm and a capacity of 10 cc. The suspension is kept gently stirred by bubbling moist air up one side of the chamber. Constant temperature is maintained by immersing the chamber in a water bath provided with plate glass windows.

The success of the method depends largely upon obtaining a well defined diffraction pattern. This requires that the sample of cells to be measured be fairly homogeneous as to size, and the suspension be of suitable density.³ The fifth minimum (counting from the image of the slit) and the maxima on either side of it, i.e. the fourth and fifth maxima, were selected for measurements since they were the farthest bands which were always clearly visible. Satisfactory measurements for any of these bands, including setting of cross-hairs and reading of scale, may be made at the rate of 10 per minute. The maximum scatter of the individual measurements is usually less than 2 per cent and the average of ten measurements has always been found reproducible within 0.8 per cent, usually within less than 0.5 per cent.

Calibration—From the measurement of the diffraction pattern the average diameter of the cells may be computed by Equation II. The values of the factor C may either be calculated or obtained directly by measuring the diffraction pattern from cells of known size. In the present experiments we have chosen the method of direct calibration.⁴

³ The eggs from any one female are relatively uniform in size, but there is considerable variation from animal to animal. It is therefore inadvisable to use mixed lots from different animals.

The optimum suspension density is best found by trial. Too few cells do not diffract enough light, while too many fog the diffraction pattern. (Differences in suspension density, however, produce no detectable change in the positions of the diffraction bands.) In our experiments, where the suspension chamber was 5 mm in thickness, the optimum concentration was approximately 20,000 eggs per cc.

⁴ Calculation of C , as may be seen by reference to Equations I and II, involves the wave length of the monochromatic light used, the scale distance, and the values of $\frac{z}{r}$ given by Millar. We have compared values of C thus calculated with those obtained by direct calibration. The two sets of values agree to within 5 per cent, the values obtained by direct calibration being consistently higher than those calculated.

TABLE I

A representative calibration experiment Samples of *Arbacia* eggs in equilibrium with five different concentrations of sea water were measured by the diffraction method, and directly under the microscope Each scale reading of the diffraction pattern is the mean of ten individual settings of the cross hairs, each microscope measurement gives the mean diameter of 50 cells It is seen that for a given diffraction band the calibration factor does not vary significantly with size of the cell

Diffraction band	Fifth minimum					Fourth maximum				
Concentration of sea water <i>per cent</i>	100	90	80	70	60	100	90	80	70	60
<i>s</i> = scale reading of diffraction pattern	734	717	697	660	633	666	644	625	590	566
<i>d</i> = microscope measurements <i>in micra</i>	76.9	79.2	82.5	85.9	90.8	76.9	79.2	82.5	85.9	90.8
<i>C</i> = (<i>s</i> × <i>d</i>) = calibration factor, × 10 ⁻⁴	5.64	5.68	5.76	5.67	5.74	5.08	5.10	5.16	5.08	5.14
<i>C</i> (Average)	5.70 × 10 ⁴					5.11 × 10 ⁴				

To this end we have prepared cells of different size by allowing eggs to swell to equilibrium in several different dilutions of sea water Measurements were then taken of the diffraction patterns (ten settings on each band) and fifty cells from each sample were measured directly by the microscope and filar micrometer A representative calibration experiment for the fifth minimum and the fourth maximum is shown in Table I It will be noted that the calibration constant *C* does not vary significantly with the size of the cells The average calibration factor is used to compute the average diameter in micra, of cells of unknown size from measurement of their diffraction pattern ⁵

Applicability of the Law of Boyle Van't Hoff Volume of Osmotically Inactive Material

From evidence presented in a recent review (1) it may be concluded that under optimal conditions the volume of a living cell in equilibrium with a solution of given osmotic pressure closely obeys the law of Boyle van't Hoff, providing that cell volume is corrected for osmotically inactive material A cell of volume *V*, in equilibrium with a solution

⁵ Repeated determinations of the average calibration factors agreed within 0.4 per cent for any one observer while the values obtained by the three different observers varied less than 1.0 per cent

of osmotic pressure P , is governed by the relation

$$P(V - b) = \text{constant} \quad (\text{III})$$

where b is the volume of the osmotically inactive material

In the case of the egg of *Arbacia* we have reported three experiments (9), based upon direct measurements, which showed that the law of Boyle-van't Hoff is obeyed by these cells, and that b occupies on the average 11 per cent of the total cell volume (7, 12, and 13 per cent, respectively, in the individual experiments). Numerous experiments with the diffraction method have confirmed our previous findings, at the same time we have obtained more extensive data on the amount of the osmotically inactive material.

In each of the present experiments approximately equal numbers of eggs from a single animal were distributed in 200 cc quantities of several concentrations of sea water, in which they were kept for from 3 to 5 hours at a temperature of about 15°C.⁶ The bulk of the supernatant fluid was then removed to give suspensions of appropriate density, and the cells were measured in the diffraction apparatus. The average diameter of the cells was computed from the mean values of ten settings each on two diffraction bands.

According to Equation III, which expresses Boyle's law, the volume of an osmotic system is a linear function of the reciprocal of the osmotic pressure. In Fig 2 it is shown that this relation holds for the living *Arbacia* egg. Here the observed equilibrium volumes from three representative experiments are plotted against the reciprocals of the corresponding concentrations (*i.e.* osmotic pressures) of sea water.⁷ Eight additional experiments in which cells were measured in at least four concentrations exclusive of 60 per cent sea water substantiate this finding. The present experiments therefore confirm our previous

⁶ Preliminary experiments had shown that under these conditions cells attained osmotic equilibrium.

⁷ There was evidence of injury (slight escape of pigment) in some of the samples of cells in equilibrium with 60 per cent sea water. This injury we attributed to the stirring of the swollen cells in the diffraction apparatus. It seemed best therefore to exclude all equilibrium volumes obtained in this concentration from the computations, though they are recorded in the graph.

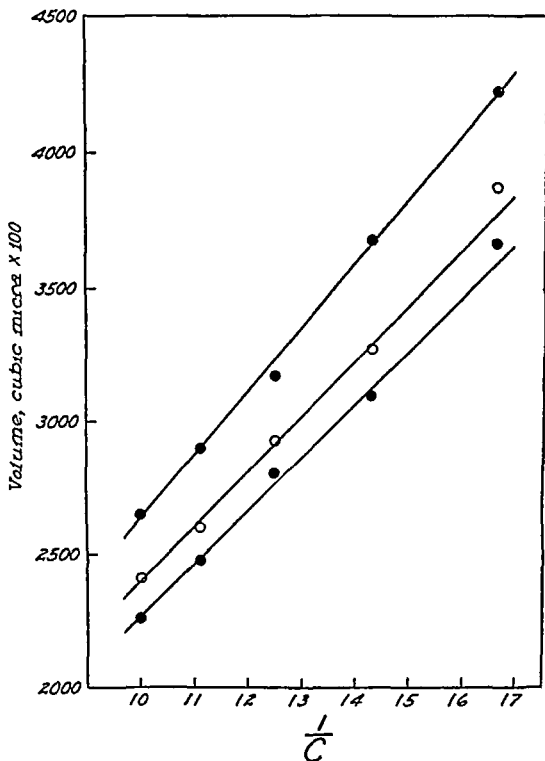


FIG 2 Results of three representative equilibrium experiments Volumes as ordinates in hundreds of cubic micra are plotted against reciprocals of concentrations of sea water (concentration of ordinary sea water = 1.0) In each experiment the line through the observed points is that computed by the method of least squares for the first four points, the volumes corresponding to 60 per cent sea water ($\frac{1}{C} = 1.67$) having been neglected because in some experiments this concentration appeared to produce injury The volumes of osmotically inactive material in these experiments are respectively, 271, 348, and $282 \times 10^3 \mu^3$

conclusions that osmotic volume changes of the *Arbacia* egg follow the course predicted by the law of Boyle-van't Hoff

From a graph such as shown in Fig 2 may be found the volume, b , of the osmotically inactive material, in a given experiment b is the intercept of the graph with the axis $\frac{1}{c} = 0$ (this extrapolation is not shown in the graph) We have made in all twenty determinations of b on egg cells from different urchins The values obtained range from 6 to 20 per cent of the initial volume of the egg, with twelve of the determinations lying between 10 and 14 per cent The average for all twenty experiments is 12 per cent, this is in good agreement with the value of 11 per cent found previously

To test the reliability of the method and the consistency of the results four of the above experiments were done in duplicate In each pair of determinations the observed volumes were in close agreement, the greatest difference corresponding to a difference in diameter of 0.5 micra, the duplicate values of b were 11 and 7, 9 and 12, 20 and 19, and 16 and 18 per cent of initial volume These figures indicate that the value of b may be appreciably affected by slight experimental errors, but we believe that the observed differences in the amount of b , ranging from 6 to 20 per cent, probably represent significant variations in the amount of osmotically inactive material in eggs from different animals

Kinetics of Osmosis Permeability of the Cell to Water

In previous studies of the course of osmotic swelling and shrinking, measurements of individual cells were made at intervals of 1 minute, in order to obtain reliable averages it was frequently necessary to repeat such experiments a number of times (10) It is one of the advantages of the diffraction method that the average size of large numbers of cells may be obtained rapidly and with satisfactory accuracy, also, these cells may be kept in suspension and stirred constantly during the experiments

The application of the method to the course of volume change during endosmosis is as follows As the first step the average size of the cells in 100 per cent sea water is determined as has already been described A concentrated suspension of the same cells is then mixed with diluted

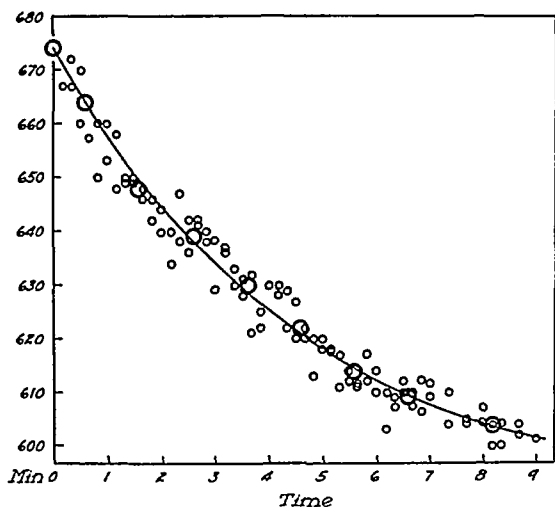


FIG 3 Measurement by diffraction method of cells swelling in 60 per cent sea water Ordinate readings are readings on the scale of the position of the fourth maximum, for which the calibration factor is 5.11×10^4 The experiment is in duplicate The small circles represent individual readings at intervals of 10 seconds the large circles are averages of these points for each minute It will be noted that in accordance with Equation II, the scale readings diminish as the cells increase in size

sea water in such proportion that the final suspension will be one of appropriate density in a known concentration of sea water⁸ This suspension is quickly introduced into the chamber of the apparatus and change in size of the cells is followed by making readings of the

⁸ By this means initial size of a given sample of cells may be obtained prior to measuring the same sample during the course of swelling or shrinking This is not feasible in direct measurements based upon small numbers of cells As a matter of fact the number of cells measured by the diffraction method is so large that no significant error is introduced if initial size and course of volume changes are determined with different samples of cells from the same animal

diffraction pattern at intervals of 10 seconds. A representative experiment illustrating swelling of *Arbacia* eggs in 60 per cent sea water is shown in Fig. 3. In this figure the scale readings of the position of a diffraction band are plotted as ordinates against the time as

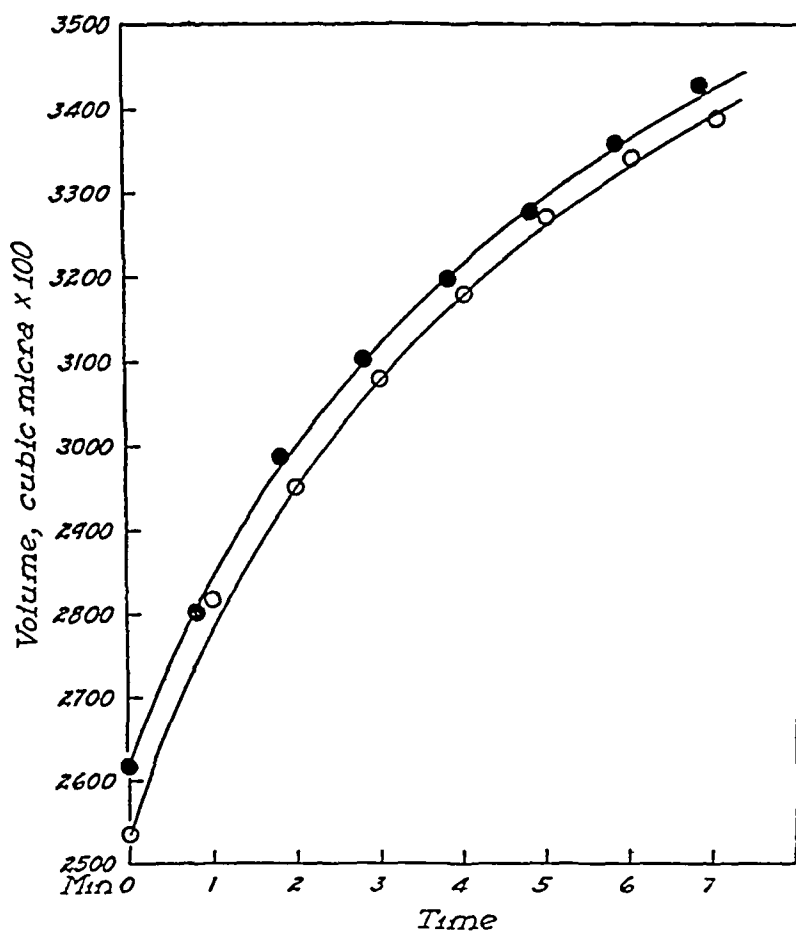


FIG. 4. Comparison of curves of swelling of cells measured in 60 per cent sea water directly under the microscope (open circles) and by the diffraction method (solid circles). The two curves are approximately parallel.

abscissae. The course of swelling is followed in duplicate for 9 minutes. The small circles give the individual readings, minute by minute averages of these points are represented by the large circles. For comparison with the direct method the minute by minute averages may be converted to volumes and plotted in the more usual manner.

Such a plot is shown in Fig 4, which also gives a swelling curve obtained by direct measurements of cells from the same animal. It is

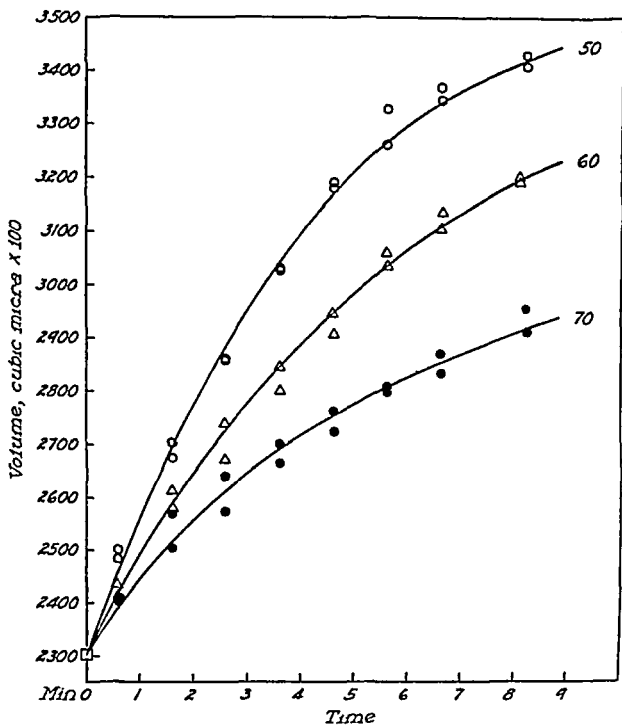


FIG 5 Curves of swelling of cells in three different concentrations (50, 60, and 70 per cent) of hypotonic sea water. Duplicate measurements are shown for each concentration.

seen that the two curves are closely parallel. The comparatively slight constant difference between the two curves, corresponding to a

diameter difference of less than 1 micron, is readily accounted for since the microscope measurements (lower curve) represent the mean of only 10 cells, while the upper curve, based on the diffraction method, represents the mean of approximately 200,000 cells

The rate of swelling of cells in hypotonic sea water of three different concentrations is illustrated in Fig 5 For each concentration the experiment was done in duplicate, the minute by minute averages are plotted separately The duplicate measurements are in satisfactory agreement, the difference in rate of swelling in the several concentrations is clearly brought out

In addition to experiments on swelling of cells, the diffraction method has also been found to be equally applicable in the study of the reverse process, exosmosis

Calculation of Permeability

Permeability of the cell to water has been defined as the amount of water that enters or leaves the cell per unit of time, per unit of cell surface, and per unit of osmotic driving force As has been shown in previous papers (10, 11) permeability may be computed from volume curves,⁹ such as are shown in Figs 4 or 5 Permeability values for

⁹ If it be desired to compute permeability from the slopes of swelling curves directly, without resorting to the use of an integrated equation, it is convenient to use the diffraction measurements without transforming them into volumes The determinations may then be made from curves such as shown in Fig 1 The differential equation for the volume curve is

$$K = \frac{dV}{dt} / S (P - P_{ex})$$

where $\frac{dV}{dt}$ is the rate of passage of water in cubic micra per minute, S the cell surface in square micra, P the osmotic pressure of cell contents, P_{ex} the known and constant osmotic pressure of the outside solution, and K is defined as the permeability Substituting for V and S their equivalents expressed in scale readings

$$V = \frac{\pi}{6} \frac{C^3}{s^3}$$

and

swelling at 22°C in 50, 60, and 70 per cent sea water were obtained in eighteen experiments by the diffraction method. These values ranged from 0.087 to 0.125, with an average of 0.106 cubic micra per minute, per square micron of cell surface, per atmosphere difference in osmotic pressure. As in previous experiments permeability was found to be independent of the external concentration. For comparison, eleven determinations of permeability by direct microscope measurements were made at approximately the same temperature as had been used in the diffraction method, the values ranged from 0.089 to 0.127, their average of 0.104 agrees with the average of the swelling experiments. Three experiments by the diffraction method on shrinking from 60 to 92 per cent sea water yielded values of permeability of 0.126, 0.124, 0.130. These recent determinations of permeability are in good agreement with the values previously reported.

Determinations of Permeability to a Solute (Ethylene Glycol)

Jacobs (12) has described a quantitative method by which cell permeability to a solute as well as to solvent may be measured. Briefly stated, the test cells are placed in a medium made by dissolving a

$$S = \pi \frac{C^2}{s^2}$$

(where s is the scale reading of the diffraction band and C the appropriate calibration factor) we obtain

$$K = - \frac{ds}{dt} \frac{C}{2s^2} / (P - P_{\infty})$$

If, for example permeability is to be determined graphically at the third minute, s is the scale reading at the instant and $\frac{ds}{dt}$ the slope of the curve at this point. P may be computed from the scale reading according to the equation

$$P = P \left(\frac{s}{s} \right)^2$$

s being the initial scale reading given by the cells in equilibrium with solution of osmotic pressure P . (This is in accordance with Equation III, neglecting b). This is the procedure that has been used in most of the computations of permeability in this paper.

desired quantity of the penetrating solute in an appropriate isotonic solution; in the present case 0.5 M ethylene glycol is dissolved in ordinary sea water) Such a solution is at first hypertonic with respect to the egg cell and hence causes shrinkage, but as the solute becomes distributed between cell and outside solution, the egg returns to its

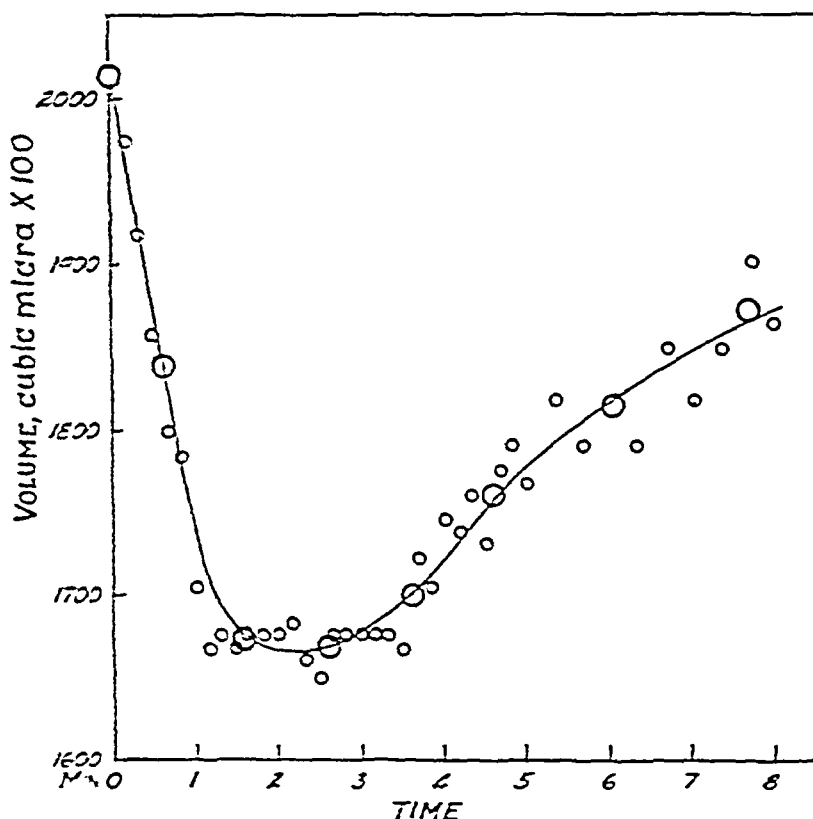


FIG. 6. Course of shrinkage and subsequent swelling of cells placed in a solution of 0.5 M ethylene glycol in sea water. Volumes corresponding to determinations made at intervals of 10 seconds are plotted as small circles; the large circles represent the 30-minute averages of these points.

original size. One of the applications of this method which has been made by Stewart and Jacobs (13, 14) is the study of the permeability of the *Arceuthobium* egg to ethylene glycol. These cells when transferred from ordinary sea water to a solution of 0.5 M ethylene glycol in sea water rapidly shrink, reach a minimum volume of approximately 80 per cent of their initial size in 2 to 3 minutes, and then slowly swell

to the original volume The data required for computation of permeability to ethylene glycol are initial size, minimum volume attained, and time of attaining this minimum Stewart and Jacobs have followed the course of these volume changes by direct measurements of individual cells As they point out, the greatest difficulty encountered in connection with this procedure is accurate determination of the initial size of the particular cell whose volume changes are to be followed This difficulty may be avoided by obtaining the average of large numbers of cells by means of the diffraction method

TABLE II

Permeability of the *Arbacia* egg to ethylene glycol Summary of four experiments Volumes are stated in hundreds of cubic micra In the last column are given the values of the permeability These values indicate that on the average 4×10^{-15} mols of ethylene glycol pass through each square micron of cell surface, per minute at a concentration difference of 1 mol per liter and a temperature of 24 C

Experiment	Initial volume	Minimum volume	Time to minimum volume	$\frac{V \text{ min}}{V}$	Permeability to ethylene glycol $\times 10^{15}$
			min		
1	2433	1979	2 50	0 813	3 9
2	2178	1788	2 50	0 821	4 1
3	2169	1750	2 83	0 807	3 3
4	2154	1781	2 28	0 827	4 7
Mean	2234	1824	2 53	0 817	4 0

We have performed four experiments on the permeability of the sea urchin eggs to ethylene glycol as follows A sample of eggs in 100 per cent sea water is measured, this gives the initial average size The suspension is allowed to settle and these same eggs are then transferred to an appropriate solution of ethylene glycol in sea water (the final concentration being 0.5 M ethylene glycol in sea water) The course of volume changes is then followed

A representative experiment is shown in Fig 6 The volumes corresponding to the individual scale readings made at intervals of 10 seconds are represented by the small circles, the large circles give the average of these points minute by minute The curve resembles that published by Stewart and Jacobs (13) The significant data of four

experiments together with the computed values of the permeability are recorded in Table II (for details of computation see (12)) The mean value of permeability of the cells to ethylene glycol in these experiments is 4.0×10^{-15} , at 24°C , that is to say, 4.0×10^{-15} mols of ethylene glycol enter the cell per minute through each square micron of surface at a concentration difference of 1 mol per liter This is in good agreement with the mean value of 4.3×10^{-15} obtained by Stewart and Jacobs at this temperature by direct measurements of 23 individual cells ¹⁰ (See Table 1 (14))

SUMMARY

1 Osmotic equilibrium and kinetics of osmosis of living cells (unfertilized eggs of *Arbacia punctulata*) have been studied by a diffraction method This method consists of illuminating a suspension of cells by parallel monochromatic light and measuring, by means of telescope and scale, the angular dimensions of the resulting diffraction pattern from which the average volume of the cells may be computed

The method is far less laborious and possesses several advantages over direct measurement of individual cells The average size of a large number of cells is obtained from a single measurement of the diffraction pattern and thus individual variability is averaged out The observations can be made at intervals of a few seconds, permitting changes in volume to be followed satisfactorily During the measurements the cells are in suspension and are constantly stirred

2 Volumes of cells in equilibrium with solutions of different osmotic pressure have been determined In agreement with our previous experiments, based upon direct microscope measurements, we have confirmed the applicability of the law of Boyle-van't Hoff to these cells, that is to say the product of volume and pressure has been found to be approximately constant if allowance be made for the volume of osmotically inactive material of the cell contents

The volume of osmotically inactive material was found to be, on the average, 12 per cent of the initial cell volume, in eggs from different animals this value ranged from 6 to 20 per cent

¹ This figure is based upon values obtained at temperatures between 23.5 and 24.4°C (See Table 1 of paper by Stewart and Jacobs (14))

3 Permeability to water of the *Arbacia* egg has been found to average, at 22°C, 0.106 cubic micra of water per square micron of cell surface, per minute, per atmosphere of difference in osmotic pressure

4 Permeability to ethylene glycol has been found to average, at 24°C, 4.0×10^{-15} mols, per square micron of cell surface, per minute, for a concentration difference of 1 mol per liter. This is in agreement with the values reported by Stewart and Jacobs

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THE THICKNESS OF THE WALL OF THE RED BLOOD CORPUSCLE

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Measurements of the impedance of erythrocyte suspensions made by Fricke (1) and McClendon (2) have been interpreted as possibly leading to the conclusion that the impedance is due to a thin layer of non conducting material of molecular dimensions surrounding the erythrocytes. Assuming a dielectric constant of 3, Fricke's calculations give a thickness of 33 \AA , which led him to suggest that the non conducting layer was unimolecular. The low conductivity would point to a layer of lipid molecules, for it is difficult to see how hydrated protein molecules could form a layer of such low conductivity.

Since any calculation of thickness from impedance measurements depends on assumptions as to dielectric constant, a discussion of the probable value of this constant for thin films becomes important. If the lipid layer is solid, 3 is a probable value, being the average value for organic substances in the solid state. If the lipid layer is liquid, as is probably the case in the erythrocyte, more factors have to be taken into consideration, for organic liquids generally have considerably larger dielectric constants.

The main contribution to the dielectric constant of naturally occurring lipoids comes from the polar groups (carboxyl, amino, etc.) of the molecules. The contribution of the hydrocarbon part of the molecules is minute. When an electric field is applied to a lipid in the solid state the electrical dipoles of the polar groups are unable to rotate the molecules as much as if the substance were liquid, and consequently lipoids have much lower dielectric constants in the solid than in the liquid state. It follows from this, however, that if the molecules in a liquid could be prevented from rotating the substance would have the same dielectric constant both in the liquid and in the

solid state. Such a condition is found in the case of a unimolecular liquid film formed at either an oil-water or an air-water interface for, though the film is liquid the polar groups are fixed in the interface and the molecules are not free to rotate. Consequently such very thin lipoid layers as e.g. that of the erythrocyte which consist of little more than two oriented unimolecular films should also have low values for their dielectric constants. This argument however is not entirely satisfactory as it stands since there will be a considerable proportion of water molecules in the interfacial films. The argument would be satisfactory if it were possible to show that the mixture of water molecules and polar groups which composes the interfacial films also has a dielectric constant of approximately 3. Fortunately an experimental proof of this can be derived from studies of unimolecular films at air-water interfaces. Films at air-water and lipoid-water interfaces are so closely similar that a direct comparison is quite justifiable so far as the polar groups are concerned.

Consider any unimolecular film. The dipole moment of each molecule in the film is μ . On the average the dipoles will be oriented at an angle α to the plane of the film. The vertical component of each dipole will therefore be $\mu \sin \alpha$. This oriented array of dipoles will produce a potential ΔV perpendicular to the plane of the film, such that

$$\Delta V = \frac{\frac{4}{3} \pi r \mu \sin \alpha}{D}$$

or

$$\mu \sin \alpha = \frac{\Delta V D}{\frac{4}{3} \pi r} \quad (1)$$

dicular to the plane of the film. Thus if a value for D is calculated on the assumption that the dipoles are vertical ($\epsilon \sin \alpha = 1$), whereas actually they are at an angle to the vertical ($\epsilon \sin \alpha < 1$), the calculated value of D will be too great. The calculated value cannot possibly be less than the true value. Consequently, if in equation (1) we write $\sin \alpha = 1$ and calculate the values of D corresponding to various values of n and ΔV , the minimum value of D obtained by this calculation will be either equal to or greater than the true value of D for the film concerned. The following table contains a series of values for D obtained with films of various substances, the data for the calculation of which have been taken from the published and unpublished work of N. K. Adam and his collaborators, J. B. Harding and J. F. Danielli. In one case, that of the nitriles, the value of D has already been published.

Compound forming film	D
Aliphatic ester	4.2
acid	5.4
alcohol	6.5
" ketone	6.4
" nitrile	6.7
" dibasic ester	3.0
Sterol alcohol	4.1
ketone	2.5

As these values are to be regarded as maximum values only, it appears improbable from this table that the value of the dielectric constant of such systems of polar groups can be much greater than 3. It would therefore appear that Fricke's assumption is justified even in the case of a liquid layer, provided the film is not more than two molecules thick. If the layer is more than two molecules thick there will be little to prevent free rotation of the molecules of the liquid layers between the two interfacial films, and the dielectric constant would be correspondingly greater than 3.

In the previous discussion the case of a continuous layer only has been considered. Similar considerations will also apply to a mosaic membrane (3), since the same factors are involved, and also to any emulsion of oil in water in which the surface area is large compared

with the volume of the oil droplets. In fact phenomena of the type discussed here must be taken into consideration in all cases in which the ratio of surface to volume is not insignificant.

SUMMARY

Fricke's assumption, that the dielectric constant of the erythrocyte wall is 3, is discussed. The assumption is approximately correct in the case of a solid layer of any thickness, and in the case of a liquid layer of not more than bimolecular thickness. For liquid layers of greater thickness the dielectric constant may be several times greater than 3. Calculated values based on experimental determinations are given of the dielectric constants of the polar groups of unimolecular films.

My thanks are due to Professor E. N. Harvey and Dr. W. Wilbrandt.

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INHIBITION OF PHOTOSYNTHESIS IN CHLORELLA PYRENOIDOSA BY THE IODO ACETYL RADICAL

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(Accepted for publication, March 20, 1935)

I

INTRODUCTION

The iodo acetyl radical interacts with the photosynthetic and respiratory systems of the unicellular green alga *Chlorella pyrenoidosa*. Before discussing these data, however, it will be convenient to review briefly certain theoretical concepts relative to photosynthesis.

It is customary to think of the process of photosynthesis as consisting of two major divisions. Various experimental procedures justify this conclusion, the following being that adopted in this paper. The method involves the use of intermittent illumination, the effect of which was investigated extensively by Warburg (1919), who placed a rotating sector before his light source. Emerson and Arnold (1932) modified this technic by eliminating the sector and making the source itself intermittent, using a neon discharge tube fired periodically by discharging a condenser through it. Thus they were able to obtain very intense flashes of light lasting about 1×10^{-5} seconds, separated by dark periods whose duration could be varied.

By means of their flashing light technic, Emerson and Arnold found the amount of photosynthesis per flash to increase with the duration of the intermittent dark periods, rather rapidly at first, then more slowly as the maximum yield *per* flash was approached. Thus in *C. pyrenoidosa* the maximum yield per flash was obtained when the intermittent dark periods had a duration of about 0.035 seconds, the experimental temperature being 25°C and saturation conditions (defined below) being maintained. As the maximum yield per flash was approached, simultaneously the sensitivity of the yield to cyanide and to temperature decreased, so that it was possible to maintain the

maximum rate by suitably adjusting the dark times, in spite of a 20°C temperature decrement or the presence of prussic acid. On the other hand, further increase of the duration of the intermittent dark periods did not compensate for low light intensity, the presence of indifferent narcotics or the inhibitory effect of brief exposures to ultraviolet light (reported subsequently by Arnold (1933)). For example, a 50 per cent inhibition of the rate in continuous light caused by ultraviolet irradiation (determined under saturation conditions), reduced the rate in flashing light by an equal percentage.

On the basis of such experiments, the photosynthetic process is divided into two parts, (1) the photochemical reaction and (2) the Blackman reaction. The photochemical reaction absorbs radiant energy necessary for synthesis, is not markedly sensitive to temperature and can be inhibited by ultraviolet light and the indifferent narcotics. The Blackman reaction is independent of light, is sensitive to changes of temperature and to cyanide, and can be eliminated as a limiting factor by the use of intermittent illumination. We may therefore regard the Blackman reaction as a process acting to restore the photosynthetic mechanism to a state which is sensitive to light and capable of binding carbon dioxide and releasing oxygen. Intermittent illumination is a device which, by allowing the Blackman reaction to run to completion, excludes it as a limiting factor. Accordingly, when the plants are saturated with respect to light intensity and carbon dioxide tension (saturation conditions), the rate of photosynthesis in continuous light is limited by the Blackman reaction. On the other hand, the photochemical reaction is limiting at low light intensities. The photochemical reaction may be made limiting under saturation conditions however by illuminating the cells with flashes of light separated by intermittent dark periods which permit the Blackman reaction to run to completion.

II

The Interaction of the Iodo-Acetyl Radical with the Photosynthetic Mechanism of Chlorella pyrenoidosa

Due to their interaction with some part of the Blackman reaction, as will be shown below, both iodo-acetic acid ($\text{CH}_2\text{I}-\text{COOH}$) and iodo-

acetamide (CH_3CONH_2) inhibit photosynthesis¹ The cause of the inhibition must lie in the iodo acetyl radical itself, since acetamide is without effect The study of these actions, however, is complicated by the fact that such compounds penetrate the *Chlorella* cell rather slowly, because moderate external concentrations do not give immediate and constant inhibitions but appear to act for 1 or 2 hours, during which the rate of photosynthesis gradually decays Such decay curves (cf Fig 1) might result also from the slowness of interaction between the iodo acetyl radical and the sensitive locus of the photosynthetic mechanism, but it would appear from the data that this is not the limiting consideration

The strongest argument in favor of the penetration explanation is that derived from the effect of pH on the action of the two poisons If iodo acetic acid be added to the cells suspended in carbonate buffer mixture IX, whose initial pH is 9.2, not even an 0.01 M concentration will cause an appreciable inhibition, whereas a similar concentration of iodo acetamide inhibits almost completely (cf Table I) But if the acid be added to Knop solution, whose pH is about 5.3, then an inhibition will be observed, though the amide, per unit concentration, is still the more effective The probable explanation of these facts rests upon the general rule that ions penetrate living cells less easily than neutral molecules of the same mass At the pH of the carbonate buffer all of the iodo acetic acid is in the ionic state, whereas at the lower pH of Knop solution an appreciable amount is undissociated A somewhat analogous case has been investigated by Collander, Turpeinen, and Fabritius (1931), who found, for instance, that ammonium acetate, which because of hydrolysis forms fewer ions, penetrates *Rhodo* cells more rapidly than ammonium chlor acetate Table I also indicates that the amide penetrates more easily from Knop solution

It is concluded, therefore, that the failure of iodo acetic acid to inhibit photosynthesis under certain conditions is the result of its failure to reach the sensitive locus, and that in general, negative results with this poison are of no significance unless its penetration can be

¹ The author wishes to express his indebtedness to Dr David Goddard, of Dr L. Michaelis laboratory The Rockefeller Institute who suggested comparing the action of acid and amide, and who prepared the freshly crystallized compounds used

established. Because it is much more convenient to study the photosynthesis of cells suspended in carbonate mixture IX (which provides saturation with respect to carbon dioxide tension), most of the experiments upon which this paper is based involved the inhibitory action of iodo-acetamide. It should be remarked also that the carbonate buffer method involves the measurement of oxygen production only.

As was pointed out in the introduction, either the photochemical or Blackman reaction can be made to limit the rate of photosynthesis by altering the experimental conditions, the cells being saturated with respect to carbon dioxide. The photochemical reaction is limiting at low intensities of continuous light, or in flashing light when the duration of the intermittent dark periods is adequate, the Blackman reac-

TABLE I

Inhibition of Photosynthesis, under Saturation Conditions at 20.5°C, by Iodo-Acetic Acid and Iodo-Acetamide

Agent added to suspending medium	Concentration of iodo acetyl radical in suspending medium	Inhibition at 30 min	Suspending medium	Initial pH of suspending medium
	molar	per cent		
Iodo acetamide	1×10^{-2}	90+	Carbonate buffer IX	9.2
Iodo-acetamide	1.5×10^{-4}	35	Carbonate buffer IX	9.2
Iodo-acetamide	1.5×10^{-4}	65	Knop solution	5.3
Iodo-acetic acid	1×10^{-2}	10	Carbonate buffer IX	9.2
Iodo-acetic acid	6.5×10^{-3}	35	Knop solution	5.3

tion is limiting at high intensities of continuous illumination. If the iodo-acetyl radical acts upon the Blackman reaction, we should expect the resulting inhibition to be decreased when the photochemical reaction tends to become limiting. That this is the case is proved by the curves of Figs. 1 and 2, which are typical of those obtained.

All of the curves of Fig. 1 result from data obtained by the use of continuous light, the differences being due to changes in the concentration of inhibiting agent or the intensity of illumination. Curve 4, for instance, involves an iodo-acetamide concentration of $1.5 \times 10^{-4} M$ at light saturation, and indicates that photosynthesis (oxygen production) was reduced 45 per cent at 35 minutes after the addition of the poison and 70 per cent after 65 minutes. Curve 3 involves a similar

concentration of amide acting upon cells from the *same* culture, which were exposed, however, to a light intensity sufficient to achieve but 50 per cent saturation. In this case photosynthesis was not reduced at 35 minutes, and but 25 per cent at 65 minutes. Thus by making the photochemical reaction limiting, the effect of the amide is masked.

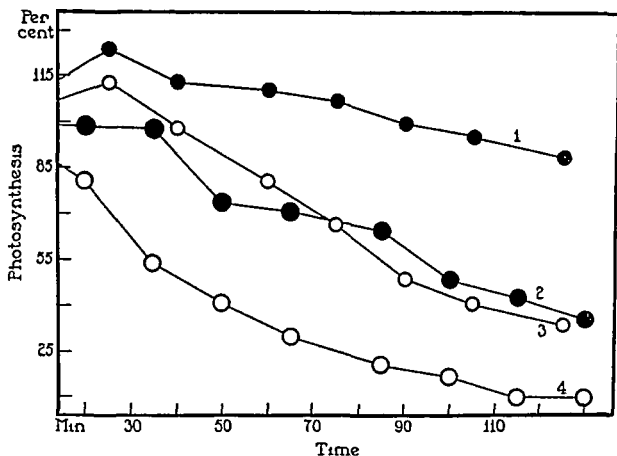


FIG 1 Iodo-acetamide inhibition of photosynthesis in continuous light. The ordinate represents the rate of photosynthesis expressed as a percentage of that for the non poisoned control. The abscissa indicates the time in minutes after the addition of the poison. The different curves were obtained by varying the concentration of amide in the suspending medium and the light saturation as follows: Curve 1, 4×10^{-5} M at 50 per cent; Curve 2, 4×10^{-5} M at 100 per cent; Curve 3, 1.5×10^{-4} M at 50 per cent; Curve 4, 1.5×10^{-4} M at 100 per cent.

Curves 2 and 1 indicate analogous results for an amide concentration of 4×10^{-5} M.

The flashing light experiments, illustrated by the curves of Fig 2, complete the proof. The first four points of the upper curve were made in flashing light, following the addition of iodo acetamide to a concentration of 1.5×10^{-4} M. The rate of flashing was 15 per second, which gives intermittent dark periods of more than adequate

duration for cells at 20.5°C, the experimental temperature. During the course of the 1st hour, the rate declined only about 15 per cent, whereas in the lower curve, made in continuous light on cells from the same culture immediately afterwards, the decrease was about 55 per

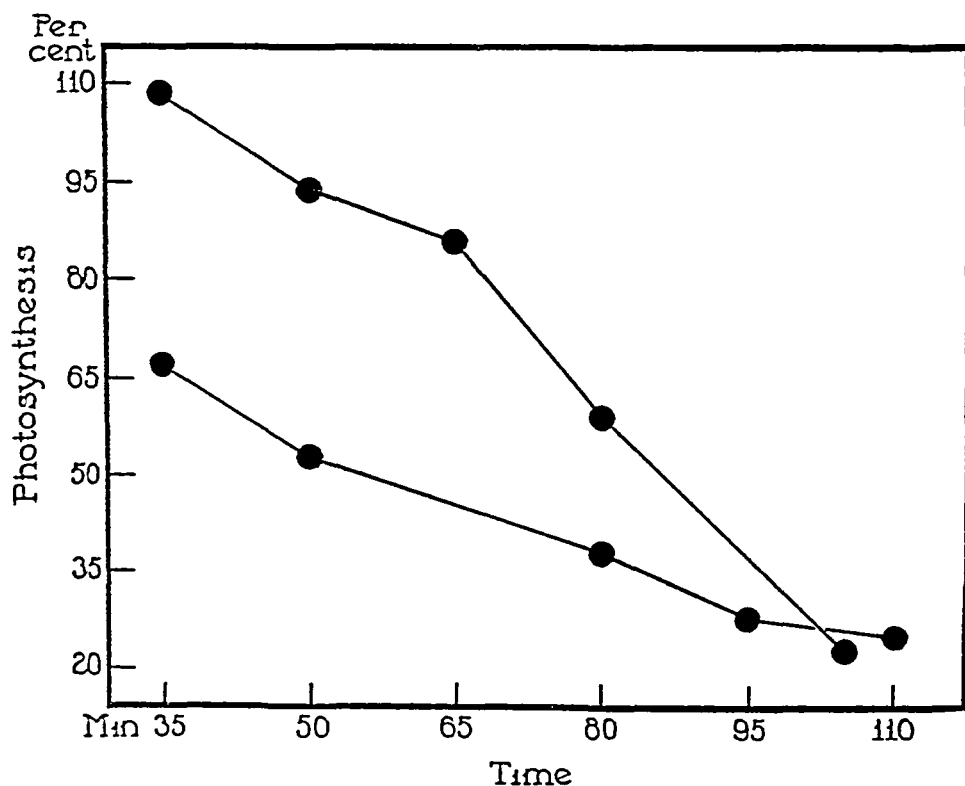


FIG. 2 Iodo-acetamide inhibition of photosynthesis in flashing light. The ordinate represents the rate expressed as a percentage of that for the non-poisoned control. The abscissa indicates the time in minutes after the addition of the poison. The concentration of iodo-acetamide was $1.5 \times 10^{-4} M$. Light saturation was maintained. The first four points of the upper curve were made in flashing light. Immediately after the fourth point, the cells were subjected to continuous illumination, the rate for which is indicated by the fifth point. The entire lower curve was made in continuous light.

cent. The flashing light curve began to drop rapidly, however, after the 1st hour so that by the eightieth minute the rate was down by 40 per cent. This is interpreted to mean that the intermittent dark periods were no longer adequate to compensate for the amount of

amide present at the sensitive locus. Following the reading at the eightieth minute, continuous light of saturating intensity was substituted for flashing light, and the next reading fell on the control curve, made throughout in continuous light. This indicates that the presence or absence of light in this experiment had little effect on the penetration of the poison. The specific experimental details are given in the next section of this paper.

Curve 1 of Fig. 1 suggests that in addition to its inhibitory action, iodo acetamide can increase the rate of photosynthesis. By using lower concentrations of amide at lower light intensities this accelerating action becomes more apparent. The increase, however, does not always appear, probably due to the fact that different cultures of

TABLE II

*Increase in Photosynthetic Rate Due to Low Concentrations of Iodo Acetamide
Light Saturation 20 Per Cent Temperature, 20 5°C*

Concentration of iodo-acetamide	Per cent control after addition of poison			
	Min			
	30	60	90	120
<i>molar</i>				
1×10^{-4}	139	135	133	127
1×10^{-5}	122	123	119	117
6×10^{-6}	130	130	123	123

cells require somewhat different concentrations of amide to produce the same effect. Table II gives the data of three cases. Such data may be inaccurate to the extent of plus or minus 10 per cent because of the low rate of oxygen production, which gives but small manometer readings, and because a correction for respiration must be applied. If this supposed increase in the rate of photosynthesis be accepted as real, it is due to the interaction of the amide with the photochemical reaction.

Just why iodo acetate should interact with the photosynthetic mechanism is a question which cannot be answered at present. Like cyanide it inhibits by way of the Blackman reaction, but this could hardly be interpreted to signify the presence of enzymes exactly similar

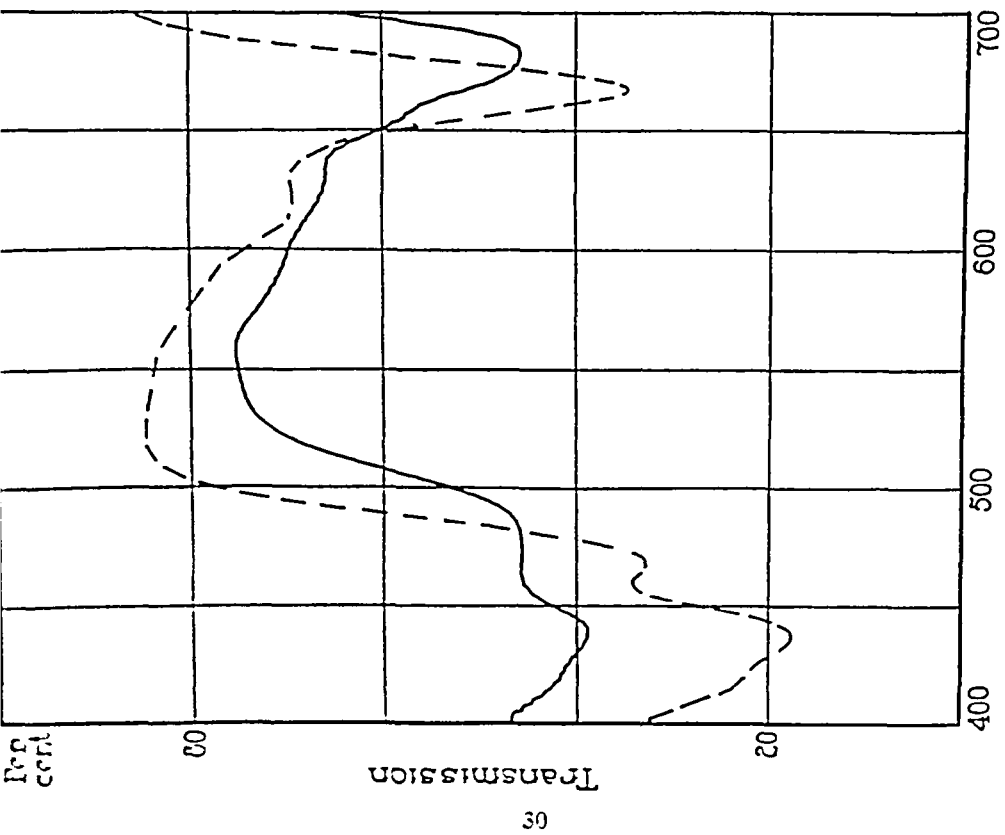


FIG 3

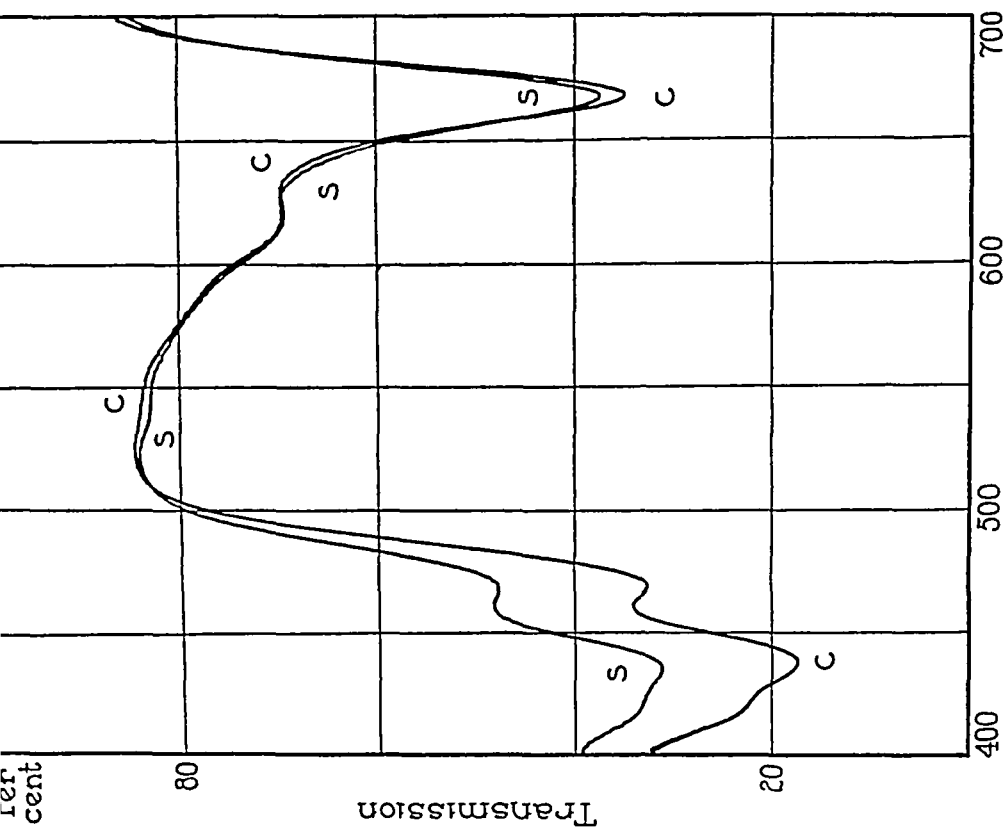


FIG 4

to those of respiration and fermentation. The work of Dickens (1933) indicates that iodo acetate reacts readily with sulfhydryl groups, Michaelis and Schubert (1934) have extended these observations, and in addition have pointed out that primary amines (amino acids), on the alkaline side of neutrality, are attacked likewise. On the other hand, it is a commonplace to associate cyanide with heavy metal catalysis. It would therefore seem probable that iodo acetate and cyanide interact with the photosynthetic mechanism at different loci within the Blackman reaction. These loci may or may not be on the same molecule.

If iodo acetate attacks chlorophyll, it would probably change the absorption spectrum of the latter. In order to check this, the absorption spectrum of a suspension of cells was determined from 4,000 to 7,000 Å u (*cf* Fig 3). Since the presence of amide (4×10^{-3} M) was without effect, the chromophore groups of chlorophyll cannot be involved, and it is likely that the same is true for the whole molecule.

For purposes of comparison, the absorption spectrum of a methyl alcohol extract of *Chlorella* cells was determined, shown also in Fig 3. The concentration of chlorophyll in this extract was made equal to that contained in the intact cells of Fig 3 by extracting an equal quantity of cells and diluting to the appropriate volume. A comparison reveals that the spectrum of the cells has only a general resemblance to that of the alcoholic extract. Not only are the peaks of absorption considerably rounded in the plant, as compared to the alcohol extract, but the absorption in the green is more, and in the blue and violet is less. The well known shift of the peaks toward the

FIG 3 Per cent transmission plotted as a function of wave length for (1) *Chlorella* cells (solid line) suspended in carbonate buffer IX and (2) a methanol extract of *Chlorella* cells (broken line). The chlorophyll concentrations are equal. To correct for the effect of the absorption cell and suspending medium, divide all transmissions by 0.9. The unbroken curve was made by the Hardy color analyzer. The broken curve was traced from one made by the color analyzer, shown in Fig 4.

FIG 4 Per cent transmission plotted as a function of wave length for (1) a methanol extract of *Chlorella* cells (C) and (2) a methanol solution of purified spinach chlorophyll (S). To correct for the effect of the absorption cell and solvent, divide all transmissions by 0.9. Both curves were made by the Hardy color analyzer.

red when chlorophyll is in the plant has been confirmed. These incidental results appear to lend support to the views of Baas-Becking and Koning (1934). To assume that chlorophyll *in vivo* is in the colloidal state, according to them, does not explain satisfactorily all of the spectrophotometric data.

For reference, the absorption spectrum of purified spinach chlorophyll dissolved in methyl alcohol is shown in Fig. 4. The greater absorption of violet light by the *Chlorella* cell extract is probably explained by the presence of xanthophyll and carotin in such an unpurified preparation.

Iodo-acetate also affects the respiration of *Chlorella*. Inhibition results from the use of concentrations higher than 1×10^{-3} M. Below this as far as 1×10^{-5} M increases of rate, as high as 100 per cent, have been obtained. The difference in threshold concentration for inhibition between respiration and photosynthesis indicates the fundamental independence of these two processes.

III

EXPERIMENTAL

Cells from the strain used by Emerson were cultured in Knop solution according to his directions (1929), and grown in an incubator, maintained at 20°C, over several neon tubes. A mixture of 5 per cent carbon dioxide in air was bubbled through the cultures continuously. Since old cultures show a declining rate of photosynthesis per unit volume of cells, none was used in which the concentration exceeded 15–20 cmm per 10 cc of culture medium. These healthy cells will produce, under saturation conditions at 20°C, somewhat better than their own volume of oxygen in 5 minutes, and this figure may be used as a factor to reduce the percentages of Figs. 1 and 2 to absolute amounts. The respiration of such cells, when determined in carbonate buffer IX, is from 5 to 10 per cent of their photosynthesis.

Photosynthesis was measured by the Warburg manometric method. This may be accomplished in two ways. The easiest is to suspend the cells in one of a number of carbonate-bicarbonate buffer mixtures, the properties of which are described by Warburg (1919). Potassium instead of sodium salts were used, however, an innovation due to Emerson and Arnold (1932). The chief action of the buffer mixture is to hold the carbon dioxide tension constant, so that the manometer measures the oxygen output of the cells. The buffer mixture used, IX, is composed of 15 parts of potassium carbonate plus 85 parts of potassium bicarbonate the concentration being 0.1 M. This saturates the cells with respect to carbon dioxide tension and has, in the case of the sodium salts, a carbon dioxide

concentration at 20°C of about 8.5×10^{-5} M, which would be in equilibrium with an atmosphere of approximately 0.002 to 0.003 carbon dioxide (Clark, 1928)

In order to determine the exact amount of photosynthesis a correction for respiration must be applied. The amount of gas exchange in the light is measured therefore, and to this is added that which occurs in the dark. The underlying assumption is that respiration is not affected by the amount of illumination.

The photosynthesis of cells in Knop solution may be measured, after equilibrating the suspension with a mixture of 5 per cent carbon dioxide in air by applying the suitable vessel constant, K , which is obtained from the following formula in which k_{O_2} and k_{CO_2} are the usual vessel constants

$$K = \frac{k_{O_2} k_{CO_2}}{k_{CO_2} - k_{O_2}}$$

For purposes of intermittent illumination, a neon discharge tube was placed in the constant temperature bath immediately below the Warburg vessels. This tube was fired by condenser discharges regulated by a commutator, turned on the shaft of a synchronous motor. The circuit is essentially that published by Emerson and Arnold (1932), and the author is indebted to Mr. W. A. Arnold for the use of the apparatus.

The absorption spectra were made at the Color Measurements Laboratory of Massachusetts Institute of Technology, by means of the color analyzer available there. This machine, designed by Prof. A. C. Hardy, is a recording photoelectric spectrophotometer and will plot, for instance, transmission as a function of wave length between 4,000 and 7,000 Å u. in 3 or 4 minutes. The color analyzer is a null instrument, and its measurements are independent of the intensity and quality of the light source, the sensitivity of the photoelectric cell, and the gain in the amplifying circuit. The transmission recorded for any given wave length in the *present* experiments is really the average for a band of 100 Å u. centered at that wave length. For further description of the instrument, the reader is referred to the article by Nutting (1934).

The depth of the absorption cell used was 1 cm., and the concentration of *Chlorella* cells used was 1 c.mm. per cc. suspended in carbonate mixture IX. The transmissions recorded are uncorrected for those of the absorption cell and suspending medium so that to obtain the true transmission of the algae, all readings must be divided by 0.9. This correction must be applied also to the curves for *Chlorella* and spinach chlorophyll. The solution of *Chlorella* chlorophyll was made by extracting 25 c.mm. of cells suspended in less than 0.5 cc. of Knop solution with absolute methyl alcohol for 2 hours. The colorless debris was centrifuged off, and the solution diluted with methanol to 25 cc. This solution of extracted chlorophyll was therefore of approximately the same concentration as the suspension of cells examined. The methyl alcohol solution of spinach chlorophyll was diluted so that it would be approximately the same concentration as that of the *Chlorella* chlorophyll.

CONCLUSIONS AND SUMMARY

Photosynthesis in *Chlorella pyrenoidosa* is inhibited by iodo-acetic acid and iodo-acetamide, both of which attack the Blackman reaction. Since acetamide is without effect, the iodo-acetyl radical must be responsible. The study of the action of the acid is complicated by the fact that its ions penetrate slowly, if at all, so that negative results with this agent are without significance unless penetration can be established. The absorption spectrum of the cells is not affected by concentrations of iodo-acetamide which completely inhibit photosynthesis. This establishes that the chromophore groups of chlorophyll are not involved, and renders it unlikely that any other part of the molecule is. Inasmuch as cyanide likewise inhibits by way of the Blackman reaction, it would seem necessary to postulate that this complex can be attacked at two different loci, which may or may not be on the same molecule.

The presence of the iodo-acetyl radical also gives rise to three other effects. (1) Concentrations (10^{-5} M or less) too small to inhibit photosynthesis may increase the rate by interacting with the photochemical complex. (2) Concentrations (*ca* 10^{-4} M) which inhibit photosynthesis increase the rate of respiration. (3) Concentrations (10^{-3} M or more) higher than those required to inhibit photosynthesis inhibit respiration.

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THE ESCAPE OF HEMOGLOBIN FROM THE RED CELL DURING HEMOLYSIS

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Recently one of us published preliminary observations on the rate at which pigment leaves the human red cell when it is hemolyzed by moderate concentrations of saponin (Ponder, 1934 *a*). Shortly afterwards Fricke (1934) gave a theoretical treatment of the subject, and showed that for a completely permeable membrane the time for the hemoglobin to decrease to 10 per cent of its original concentration should be 0.16 second in the case of the human erythrocyte. The longer times (several seconds) observed when moderate concentrations of saponin are used must accordingly correspond to a state of partial permeability, and in view of the results of the theoretical treatment it is desirable to have complete experimental data. With these this paper is concerned.

Methods

Human red cells were used because of their large size and the rate of escape of the pigment was found, as before, by taking moving pictures of the fading cells and making measurements on the films. In order to get a large number of cells per frame, the magnification was only 264 instead of 860, as in the preliminary measurements and more attention was paid to obtaining good contrast than to having a high degree of resolution with its attendant disadvantage of small depth of focus. High resolution was in fact, out of the question when the mixing of the cells and the lysin was done with micro pipettes.¹

¹ Principally because, in order to obtain working room, the cover glass on which the drops of suspension are placed has to be mounted a considerable distance above the condenser, which must either be a long focus condenser of correspondingly small aplanatic aperture, or an ordinary condenser with its top lens and consequently most of its efficiency, removed. Under such circumstances nothing is to

Two methods of mixing the cells and the lysin were used, according to whether the latter was dilute or concentrated. In the former case, a small drop of a 5 per cent suspension of washed red cells in 1 per cent NaCl was mixed on a slide with an equal drop of saponin of known concentration, also in 1 per cent NaCl. A slow frame speed (5 per second) can be used in the interests of economy. The cells can easily be kept in focus by observing them through the side telescope with which the apparatus was fitted, and this procedure is quite adequate provided the lysis of the majority of the cells takes 30 seconds or longer. The lysin, of course, is diluted to half its original concentration in the process of mixing.

If hemolysis is more rapid the mixing must be done with a micro pipette. A number of small drops of the suspension, less than 1 mm in diameter, is placed on a cover-glass with a fine capillary pipette. The cover-glass is then inverted over a moist chamber and a drop focussed. The lysin is drawn up into a micro pipette with a tip about 10μ wide, and a small droplet is injected into the middle of the drop of suspension. The droplet of lysin rapidly spreads outwards, pushing the cells before it, and those at the interface are acted on by the lysin in virtually full concentration.² Cinematography is begun as soon as the micro pipette is raised into the drop of suspension, and continued until hemolysis is complete. Relatively rapid frame speeds (15 to 25 per second) are necessary. It is remarkable how the cells remain in the plane of focus, and lysis is preceded by a short latent period during which the interface can be brought to the centre of the field. The procedure can be repeated as often as there are drops of suspension on the cover-glass.

If the rate of escape of pigment during hypotonic hemolysis is to be measured, small quantities of NaCl of different tonicities, or even of water itself, are injected into still smaller droplets of concentrated suspension. Under these circumstances it is very difficult to keep the cells in focus, and such as appear in focus on the film do so by pure chance. Further, it is impossible to be certain what the tonicity really is after the mixing, and so such experiments yield only very approximate results.

be gained by using an objective of a high n_a , and, when the mixing has to be done with micro pipettes, it is very doubtful if the effective n_a of the system can be raised above about 0.5. When the mixing is done by adding the lysin to the cell suspension and covering in the usual way, the n_a can be raised to at least 0.9, with a corresponding improvement in resolution.

² Some dilution must, of course, take place, but as the volume of the injected drop and that of the drop of suspension are about equal, the greatest possible dilution is about 1 to 2, and it is very unlikely that a dilution as great as this occurs in the neighborhood of the interface. Even if it did, the general course of the graph shown in Fig. 1 would be little altered, and failure to take account of such dilution as occurs would tend to make the experimental fading times in the higher lysin concentrations a little longer than they really are.

The developed film is driven through a "Moveola," and, the frame speed being always known one can fix one's attention on a single cell and measure the time between the moment when it begins to fade and the time when it contains only about 10 per cent of its initial amount of pigment. The beginning of the fading process is very sharp. Shortly before hemolysis occurs, each cell becomes spherical and stands out with great distinctness from its background. Because the concentration gradient of the pigment is at a maximum initially, the rate of escape of pigment is greatest when fading begins, and the passage of the large amount of hemoglobin across the cell surface gives the cell the appearance of slightly increasing in size principally because of the sudden change in the intensity of the diffraction bands surrounding it. This change can be particularly well seen with the Cassegrain dark ground condenser (Millar, 1925).

The moment at which the cell contains only 10 per cent of its initial hemoglobin is more difficult to fix, and the only practical way of determining it is to make a visual comparison of the density of the image of the fading cell with that of images on spherical but unhemolyzed cells and of spherical cells which are known to contain about 10 per cent of the pigment initially present. To obtain the photographs for comparison one proceeds as follows. To 1 cc. of packed cells is added 9 cc. of a NaCl solution so hypotonic as to produce about 95 per cent hemolysis. The concentration of pigment in the hemolyzed ghosts of such a suspension is then approximately one tenth of that initially present in the cells. Sufficient 10 per cent NaCl is added to restore the original tonicity, under which circumstances the so called "reversal of hemolysis" occurs, *i.e.*, the ghosts shrink to the same volume as that normally occupied by the cells and become visible against their background (see Bayliss, 1924-25). The 5 per cent of cells which did not hemolyze also assume their original volume, and these contain their original hemoglobin in its original concentration while the concentration in the ghosts is either 0.1 or about 0.13 of the original, according to whether the cell membrane is permeable or impermeable to hemoglobin after the "reversal," with its concomitant shrinking, has occurred, a point which is not known. The cells and ghosts are gently centrifuged off, lecithinated in order to convert them into spheres (Ponder and Robinson 1934), and at once photographed. Since they are seen side by side on the developed negative one can form an excellent estimate as to how pale a cell looks when it has lost 90 per cent of its pigment. It looks surprisingly pale. With this estimate to guide one, it is then possible to fix fairly exactly the moment during the fading process at which a 90 per cent reduction in hemoglobin concentration is reached.³

³ At first sight it might appear that densitometry would be an improvement over mere visual inspection but there are several grave objections to density measurements on these films. (1) The image of the cell is small and the resolution poor (see footnote 1), as a result the image is largely made up of diffraction bands whose intensity and position are unknown. (2) Absorption of directly incident light by the pigment contained in the cell is not the only factor which

An approximate idea of the "fading time" in different concentrations of lysin having been obtained by viewing the film in the "Moveola," portions of the film can be examined with a lens, frame by frame, and an even closer value for the rate of escape of the pigment arrived at

RESULTS

The results for saponin and human red cells are shown in Table I and in Fig 1, each value being an average for from eight to sixteen cells. The table gives the dilution of saponin in the entire system, the time t for fading to a 10 per cent hemoglobin concentration, the permeability of the membrane to hemoglobin, μ_H , and the product Nd , from which the necessary number N of holes of diameter d can be

TABLE I

Dilution, 1 in	Fading time t	$\mu_H \times (10^{-4})$	Nd
	<i>sec</i>	<i>cm/sec</i>	(<i>d in μ</i>)
25,000	11.6	0.187	0.26
20,000	11.5	0.189	0.27
18,000	11.0	0.198	0.28
16,000	9.0	0.243	0.34
14,000	6.0	0.369	0.52
10,000	3.6	0.625	0.87
5,000	1.5	1.61	2.2
1,000	0.55	5.50	7.7
100	0.23	30.7	43
10	0.20	53.8	75

found if one is considering the hypothesis that lysis occurs because of the appearance of holes in the membrane. In calculating μ_H , Fricke's simplified expression (4) was used, and this assumes that the fading time is long in comparison with 0.16 second, the time corresponding to

determines the density, for a considerable quantity of light is scattered at the cell surface. (3) The density of the image is exceedingly sensitive to small differences in focus. We have tried to make density measurements on films in which fading cells were photographed at high magnification, and the results have proved quite unsatisfactory. It is possible to show that the decrease in density is approximately linear with time t ; that the fading is exponential, but the scatter of individual points about the line leaves one very dissatisfied with the whole procedure.

a completely permeable membrane. For the shortest experimental fading times this condition is not satisfied, but for our purpose it is sufficient to subtract 0.16 second from the experimental fading time, and this has been done throughout. The value 2.8×10^{-4} cm. was used for ρ , and 7×10^{-7} cm²/sec. for D .

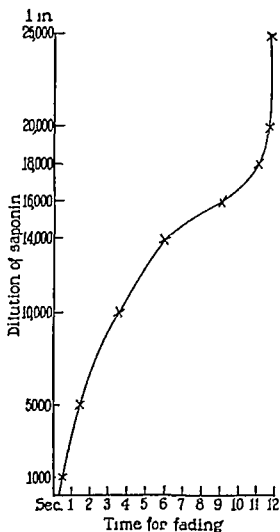


FIG 1

Extrapolating to zero dilution, the fastest fading time would be 0.195 second, which is a little longer than the theoretical time corresponding to complete permeability of the membrane, 0.16 second.

The experiments with hypotonic saline, while much less satisfactory, were definite in showing that the time for fading is virtually independent of the tonicity used to bring about lysis. If a cell hemolyzes at all, the loss of pigment occurs in from 0.6 to 0.8 second, which corresponds to quite a high permeability of the membrane to hemo

globin ($\mu_H = 4.9$ to $3.4 (10^{-4})$ cm /sec) It is very difficult, however, to ascertain the fading time with any degree of exactness, principally because the cells are swollen before they begin to hemolyze, and also because their form is not regular

DISCUSSION

These results are best discussed under a number of separate heads

1 The most important point is that in the case of hemolysis by a substance like saponin the permeability of the cell membrane is a function of the concentration of lysin used. When the latter is very great, the fading time is such as corresponds to a state of very high permeability of the membrane to hemoglobin, while when the lysin concentration is small, the permeability may be less than 1 per cent of the maximum value observed. Even with the greatest lysin concentration used, however, the fading time is appreciably longer than that which corresponds theoretically to complete permeability, but this may be due to one of two things. (a) It will be shown below that much of the increase in permeability in the higher saponin concentrations probably occurs within the short fading time itself, and it is likely that the cell begins to fade when the minimal value of μ_H (about $0.185 (10^{-4})$ cm /sec) is reached, the permeability then increases rapidly and the cell may be completely permeable to pigment before fading is complete, but the maximum permeability observed will, under such circumstances, be always a little less than that which would be observed if, during the entire fading time, the permeability were complete. The delay is probably about 0.02 second and subtraction of this value from the shortest fading time observed brings the latter still closer to the theoretical value. (b) Since the cell envelope as a whole is thicker than the thin layer upon which permeability depends and since the former does not wholly disintegrate, some barrier to the diffusion of hemoglobin may still exist even when the latter layer is completely broken down. The barrier, however, cannot be a very substantial one, for the maximum value of μ_H observed differs from the maximum theoretical value by only about 10 per cent. Virtually all the impermeability must therefore reside in the thin layer just mentioned and the 10 per cent difference might easily be accounted for by supposing the pigment to have to diffuse across a gel-

like "envelope" When the lysin is very dilute, on the other hand, we approach a minimal value for μ_H (about 0.185 (10^{-4}) cm/sec), and, unless the lysin can bring about this degree of permeability, the cell does not hemolyze at all. There is a correspondence between these results and those obtained from measurements of electric conductance, for when saponin is used in low concentration the cells, although hemolyzed, are found to have a conductance too low to measure, whereas saponin (and some other lysins) in high concentration produces a complete permeability to the electric current (Fricke and Curtis, 1935).

2 It is important to consider an apparent incompatibility between this dependence of permeability upon lysin concentration and the idea that the lysis of a cell occurs when a certain fixed quantity of lysin has been utilized in combining with the cell envelope (Ponder, 1934 *b*), particularly as the latter idea underlies the present treatment of the kinetics of hemolytic systems. The apparent contradiction disappears when we consider the quantity of lysin which is used up in combining with the cell envelope during the time for fading itself, and the point is best illustrated by a numerical example, applicable to the systems used in the foregoing experiments.⁴

Suppose complete lysis to occur in infinite time in a dilution of 1 in 25,000 (80 microgm in the entire system), the least resistant cells of the suspension may then be expected to hemolyze when about 8 microgm of lysin is present, and it is these cells which are usually observed in the films. In the usual equation for the velocity of transformation (Ponder, 1934 *b*, expression 30), put $n = 2.0$ and $k = 10^4$, this gives a time of 9 min for the lysis of the least resistant cells in a saponin dilution of 1 in 20,000, which is about the right value. Retaining these values of the constants, let us calculate how much lysin will enter into combination with the cell envelope during the times taken for fading. The results are shown in Table II.

More specifically, suppose that a cell begins to hemolyze in a 1 in

⁴ The kinetics of hemolysis in systems such as these are so well known that it is easy to select approximately the right constants. It is doubtful, of course, whether the usual equations really apply to such great saponin concentrations as 1 in 10 and 1 in 100, but the calculated values may be taken as illustrative of the point under discussion.

20,000 dilution of saponin when the amount of lysin transformed in the system is 8 microgm, and that the permeability of its membrane is the minimal value of 0.185 (10^{-4}) cm/sec, the permeability will undergo virtually no increase during the time taken for loss of pigment, for the additional lysin transformed during this period is only 0.16 microgm. Were the same cell to begin to hemolyze in a dilution of 1 in 10 when 8 microgm of lysin was again transformed, its initial permeability being the same minimal value as before, there would be transformed during the short fading time of 0.2 second an additional 1500 microgm of lysin. This quantity is so great that the membrane would almost certainly be wholly disintegrated before the end of the fading time, i.e., its permeability to hemoglobin would become complete.⁵

TABLE II

Dilution, 1 in	Fading time	Lysin combined
	<i>sec</i>	<i>microgm</i>
20,000	11.5	0.16
10,000	3.6	0.40
1,000	0.55	7.0
100	0.23	200
10	0.20	1500

3 The results throw little light on the question as to whether lysis occurs because of a generalized increase in membrane permeability or because of the appearance of holes in the membrane. For the stronger concentrations of lysin, however, one would have to postulate so many holes that the distinction is almost meaningless.

4 The relative constancy of the fading time when cells are hemolyzed by hypotonic solutions is interesting in view of the way in which hypotonic hemolysis is generally supposed to occur *viz*, by the cell's swelling to a certain critical volume (Jacobs, 1930), and its surface

⁵ In a saponin concentration of 1 in 10, something of the order of 10 per cent of the cell envelope would be transformed within the time of fading. It may be pointed out that, on the hypothesis that lysis is due to the appearance of holes, the total area of the holes which it would be necessary to postulate in order to get a fading time of 0.2 second would be about 10 per cent of the cell area. There may be some connection between these two facts.

undergoing a definite amount of extension. It is not surprising that a constant amount of stretching should be associated with a constant permeability to hemoglobin, the numerical value of μ_H , however, is unexpectedly high in view of the fact that the cells, even when hemolyzed, can be treated as virtual non conductors (Fricke and Curtis, 1935). It should be remembered, on the other hand, that the observed fading times of 0.6 to 0.8 second are probably a minimum estimate, for the cells are swollen, and therefore relatively pale, when the fading begins, and there is no way of making comparison photographs to show what density corresponds to a 90 per cent loss of pigment, as there is in the case of saponin. The most that can be said is that the observed fading time, even if it is 100 per cent too short, is scarcely compatible with the occurrence of a single rent or hole in the cell membrane, i.e., the cell appears to leak pigment rather than to burst.

SUMMARY

By means of measurements from cinematograph films of the time taken for human red cells to lose hemoglobin while hemolyzing, it is shown that small concentrations of saponin bring about a relatively small permeability of the cell membrane to the pigment, whereas large concentrations so destroy the membrane that the theoretical time for loss of pigment through a completely permeable membrane (0.16 second) is very nearly attained. These results are in agreement with those obtained from electrical measurements, and the dependence of permeability on lysin concentration can be explained on the basis of what is known about the rate of transformation of lysin as it reacts with the cell envelope. When cells are hemolyzed by hypotonic solutions, on the other hand, the permeability of the membrane to pigment is nearly constant, irrespective of the tonicity used to bring about lysis.

We have to thank Dr. Robert Chambers for his kindness in letting us use his motion picture camera.

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THE REVERSIBLE HEAT ACTIVATION INDUCING GERMINATION AND INCREASED RESPIRATION IN THE ASCOSPORES OF *NEUROSPORA TETRASPERMA*

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INTRODUCTION

It has been clearly established by B O Dodge (1) and Shear and Dodge (2) that the ascospores of the fungi *Ascobolus* and *Neurospora* are normally dormant and will germinate only after they have been heated. The heat treatment (or heat activation) which overcomes the dormancy consists in heating the spores to temperatures of 50° or higher for a few minutes and then cooling to room temperature. The spores germinate 3 to 5 hours after returning to the lower temperature. No information is available in the literature as to the nature of the activation process nor to the reactions it calls forth other than the end result, germination. This paper will deal with certain phases of the activation process and the change in the respiratory rate that it brings about, in the ascospores of the fungus *Neurospora tetrasperma*.

It will be shown that the spores may be activated when all the respiration is blocked, if the respiratory block is removed shortly after the activation is completed. But if the respiration is prevented for 2 to 4 hours after activation, the spores do not germinate when returned to conditions that allow respiration, these spores are found to be dormant, in spite of the previous activation, and will germinate only if reheated. This may be repeated several times on the same spores, thus it is clear that the whole cycle of activation and deactivation is reversible. Further it will be shown that the first change that can be detected after activation, is a large increase in respiration of the activated spores. This high respiration must continue for several

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hours before germination may occur. Partially blocking this respiration delays germination and completely blocking it leads to reversible deactivation. This respiration is cyanide sensitive.

This paper will show that the effect of heating on the activation of the spores, manifests itself so abruptly in the narrow temperature range between 49 and 52°, that it is not advisable to speak of a temperature coefficient of activation, but to describe the process as one with a critical temperature at 49–52°C. Within this narrow range of temperature the percentage germination at constant temperature as plotted against the time of heating, follows an S-shaped curve, which is best interpreted as a population or probability curve.

Dormant cells and organs are well known to biologists, common types are the gametes of many plants and animals which normally are activated (stimulated to normal development) by sexual fusion, and some seeds and spores. The zygotes of many lower plants are dormant and only germinate after a "period of rest." In some cases the zygote is not dormant but undergoes a few cell divisions giving rise to several cells (spores) and these cells may be dormant. This is the case in *Neurospora*, the zygote undergoes three nuclear divisions to give rise to four or eight ascospores according to the species. These spores are always dormant in *N. tetrasperma* and most of them are dormant in the other species. The only method known to bring about their germination is the heat treatment described above.

The ascospores of *Neurospora* are not the only dormant cells that are known that respond to a heat treatment. Mild heating of the eggs of star fish (3) leads to membrane elevation. Tarr (4) has shown that the heating of certain bacterial spores leads to greater activity of their dehydrogenase systems. Other dormant cells may be activated in various ways. Dormant potato tubers (5) are stimulated to sprout by some chemicals as ethylene chlorohydrin, and sea urchin and starfish eggs are activated by hypo and hypertonic solutions, etc. (6) Warburg (7) showed that the fertilization (activation) of sea urchin eggs leads to an increase in the respiratory rate. The activation and the correlated respiratory changes have been studied in great detail by Runnstrom (8).

No case other than *Neurospora* and *Ascobolus* of dormant cells that respond to heat alone or where the temperature effect is so very

abrupt, is known to the author. Some cases of activation are partially reversible, but no example of other dormant cells that may be activated and deactivated several times, has come to the writer's attention. Although changes of respiratory rate on activation are well known, this is probably the first case of the completely reversible effect of activation, deactivation, and reactivation on the respiration.

I

The Critical Temperature of Activation

The temperature to which the spores must be heated to induce germination was determined by heating the spores for 20 minutes at various constant temperatures and determining the percentage germination 6-8 hours later. The conditions of heating and cooling to room temperature were such that there could be no considerable lag, and the bath was constant to $\pm 0.03^{\circ}\text{C}$. The same results were obtained if the spores were heated in distilled water or buffered culture medium.

The results of the experiment are shown graphically in Fig. 1. Each point represents the percentage of germination based on counting 350-500 spores, under the microscope. The fact that the activation process appears to have a critical temperature is even more obvious if we realize that changing the time of heating does not appreciably alter the position of the critical temperature. Shortening the time merely makes the curve steeper at about $52-53^{\circ}\text{C}$. Increasing the time of heating tends to decrease the steepness of the curve a little, but it does not seriously alter the position of the critical temperature. Only a few degrees below the critical temperature no spores will germinate at all, and at 1 or 2 degrees below only a small per cent of the spores will germinate.

The heat killing curve was determined in the same manner as the activation curve. It is clear from Fig. 1 that the process producing activation is not merely the first phase of the process causing heat death. The slopes of the two curves are different and there is a 10° separation between the temperature inducing full activation and the beginning of heat killing.

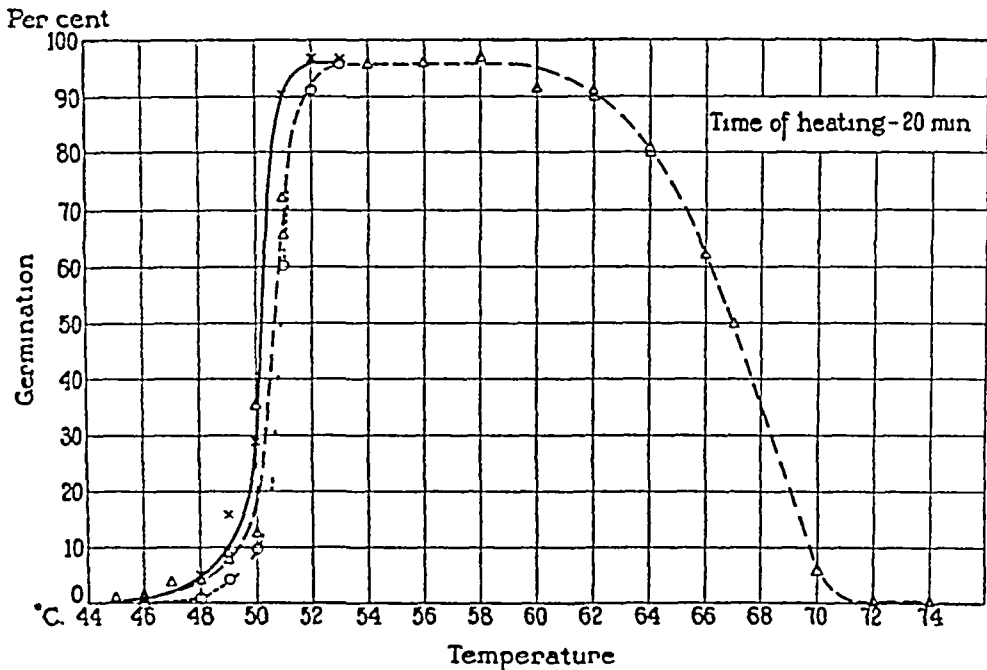


FIG 1 The effect of the temperature of activation on the percentage germination. Three separate spore lots were used for the activation, one for the heat killing. Each point is based on the counting of 350 to 500 spores.

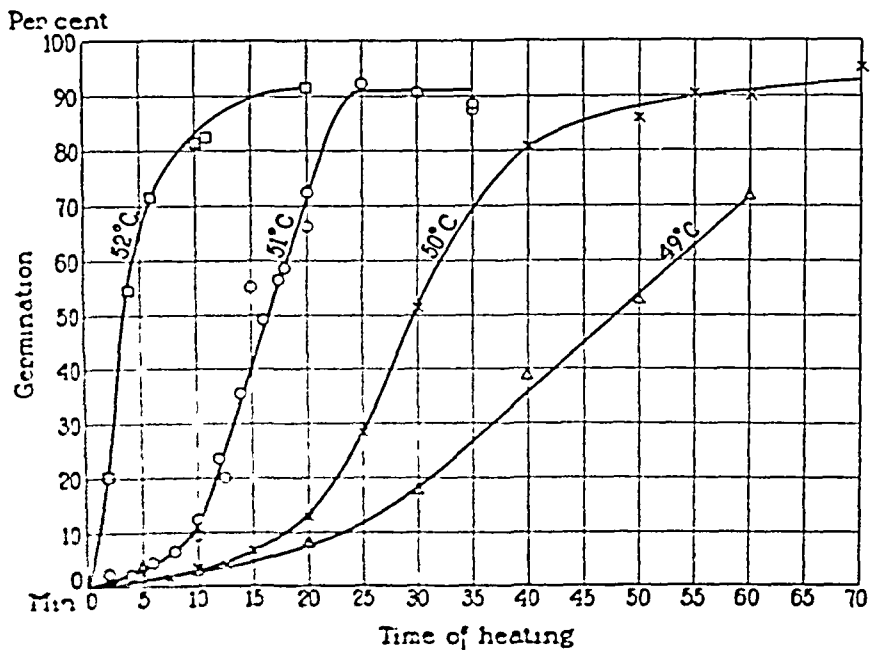


FIG 2 The effect of time of activation at constant temperature on the percentage germination. The same spore lot was used for all curves.

II

The Rate of Activation at Constant Temperature

It seemed as if it might be possible to determine the order of the chemical process underlying the spore activation, by determining the percentage germination at constant temperature while varying the time of heating. These experiments were conducted in a manner similar to the preceding ones. The temperature interval in which it is possible to follow the percentage activation against time of heating is between 49 and 52°C. At very little below 49°C some activation occurs, but regardless of the time of heating many spores never germinate. Above 52°C the activation is so nearly instantaneous that no further change with temperature can be shown until the temperature becomes high enough (over 62°C) that some of the spores are heat killed.

The data in Fig. 2 will be seen to fall on S shaped curves. These are not kinetic curves, unless one wants to interpret them as the curves of an autocatalytic process. They may equally be interpreted as a measure of the variability of the population or the variation in time which it takes the same chemical process to reach the same stage of completion, in several microscopic systems. This may be clearer if we point out that the first derivative of the curves in Fig. 2 would be normal distribution curves. But it makes no difference whether we consider a normal distribution curve as the distribution of the amount of some chemical substance in different cells or in the time at which a given process will have reached the same stage of completion. It is frequently assumed that starting with the same concentration of reactants and the same conditions that a chemical process will reach the same stage in the same time. This is true as long as we are dealing with statistically large systems, but if the systems are sufficiently small the individual systems will reach completion at different times. And these times will fall along an S shaped curve.

If we try to calculate the temperature coefficient of the activation process in the ordinary way, we obtain the results in Table I. The values were obtained from the data in Figs. 1 and 2 by applying the van't Hoff equation. For Fig. 2 the slopes of the curves at 50 per cent germination were used for the calculation. The values in Table I are

so large and their variation with temperature is so enormous that they have no meaning. The highest temperature coefficients known, those of protein denaturation (9) and of bacterial killing (10) are never of this order. We are forced to the conclusion that the activation process has a critical temperature at about 50°C. We may compare it to a change of state. Changes from solid to liquid and other comparable changes of state (phase changes) have critical temperatures. That phase changes may control some biological processes seems possible from the work of Burk (11). He found that the pH dependence of nitrogen fixation in *Azotobacter* followed a curve which seems to be best interpreted as a phase change depending upon the pH.

The activation of the spores appears to be another example of an all or none reaction, for regardless of the per cent of the spores that

TABLE I

Temperature interval	From Fig 1	From Fig 2
°C	Q ₁₀	Q ₁₀
46-48	200	—
48-49	400	—
49-50	40,000	1,360
50-51	90,000	380
51-52	—	10,000

germinate, the time between activation and germination is constant (at constant temperature). Either the spores germinate after the lapse of the normal time, or they do not germinate at all.

III

The Reversibility of Activation

The close relationship between respiration and many cellular processes suggested that the activation process might be dependent upon respiration. The method employed here was to heat the spores under conditions where the respiration was entirely blocked, and at the completion of the activation to return the spores to conditions that normally would allow full respiration. After the usual time of 6-8 hours the percentage germination was determined, and compared

with controls activated under conditions that allowed normal respiration. The results are shown in Tables II and III.

TABLE II

	Time in N after activation	Germination	Reactivated germination
	<i>hrs</i>	<i>per cent</i>	<i>per cent</i>
Control (activated in air)	—	94.2	
Activated in N	0.0	95.9	
“	0.5	96.3	
‘	1.0	96.7	
‘	2.0	74.9	
	3.0	41.1	
“ “	4.0	2.7	
	9.0	1.6	92.5
“	50.0	0.0	94.7*
‘	24.0	4.1	91.5†

* A duplicate tube was reactivated and set aside unopened for an additional 24 hours, and the data are given in the next row below.

† These spores have been three times activated and twice deactivated.

TABLE III

	Time in HCN or CO after activation	Germination	Reactivated germination
	<i>hrs</i>	<i>per cent</i>	<i>per cent</i>
Control	—	96.6	
Activated in HCN	0.0	95.6	
‘	0.5	93.7	
‘	1.0	85.6	
	1.5	75.5	
‘ ‘	3.0	52.8	94.5
“ ‘	4.0	37.4	
‘	7.0	0.0	
	Time in CO		
Control	—	94.0	
Activated in CO	0.0	96.0	
	20.0	23.0	89.0

These experiments show clearly that the activation process may occur normally when the spores are in oxygen free nitrogen, carbon monoxide, or in cyanide. But if, following activation, the spores are

maintained for several hours under conditions where no respiration is possible they have lost the ability to germinate when the respiratory block is removed. But if the spores, which have become secondarily dormant by preventing the respiration of the activated cells, are reheated they germinate and develop normally. The whole cycle of activation and deactivation is reversible several times.

Great care must be taken to avoid traces of oxygen in the nitrogen or deactivation will not occur. Low oxygen tensions prevent the germination of the spores but do not cause deactivation, since the spores will germinate normally when brought into oxygen (or shaken with air). Apparently a higher respiratory rate is essential for germination than for maintenance of the spore in the activated state.

IV

Respiration Experiments

The experiment above showed that preventing the respiration of the activated spores led to secondary dormancy. It seemed of interest to determine if the respiration of the activated cells increased prior to germination. There is a period of about 3 hours between completion of the activation process and the first sign of a germ tube which may be seen under the microscope.

The respiration was determined on the Barcroft-Warburg apparatus, with equal samples of the same spore suspension used for dormant and activated series, so that the results within an experiment are directly comparable. The spores were activated in the Warburg vessel at 54–55° for 20 minutes. Some difficulty was met in trying to get large enough amounts of ascospores (free from vegetative contamination) to perform the respiration experiments.

The curves in Fig. 3 show clearly the effect of activation on the respiration of the spores. The increase in respiration is apparent soon after activation and long before any sign of germination. A second increase in respiration occurs at the time when the first visible sign of germ tubes may be detected. The primary increase in rate of respiration is 10–15 times that of the dormant cells, and the secondary increase in rate is about 1.7 to 2.0 times. It is difficult to determine the rate of respiration of the dormant spores, because the rate is so low that it may be due to contamination by vegetative cells (particu-

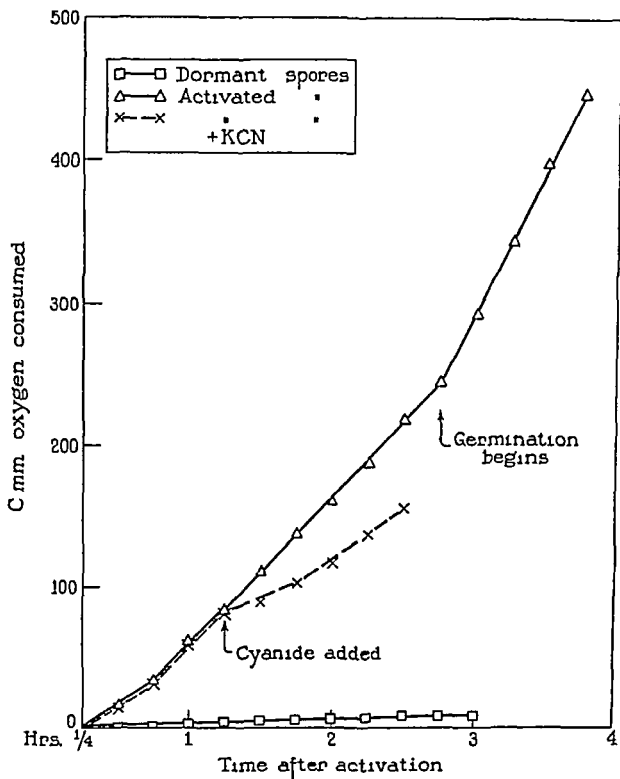


FIG 3 Influence of activation and germination on the respiration 0.046 ml of ascospores in distilled water, temperature 19.7 C zero time at completion of the activation Activated series 97 per cent germination dormant series no germination

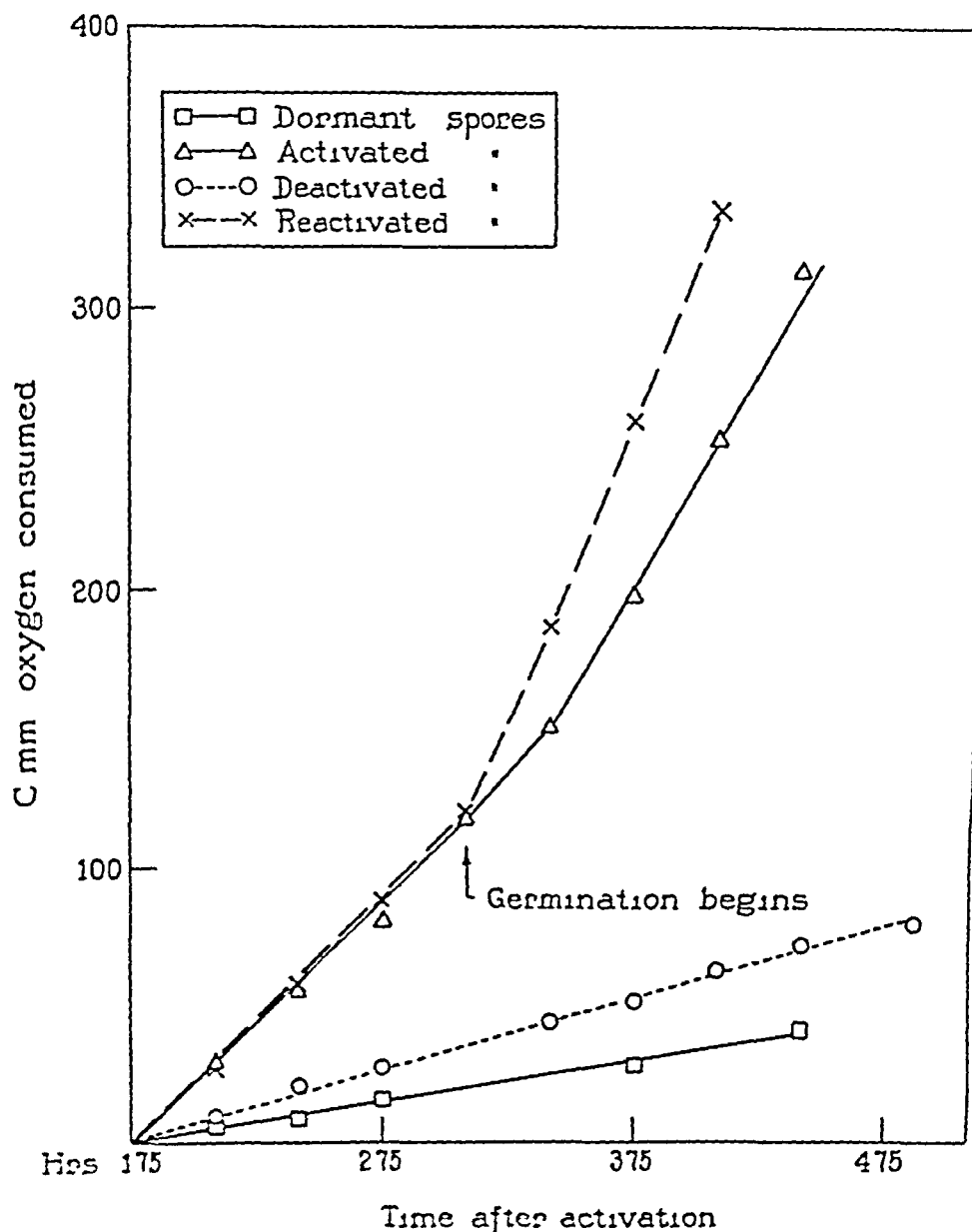


FIG. 4. Influence of reversible activation and deactivation on the respiration of the ascospores. 0.058 ml of spores, temperature 25.1°C, zero time at completion of the activation. Percentage germination: activated 83, deactivated 18, reactivated 94, dormant 0.0.

larly conidia) The first effect of activation that we here detected is then a large increase in the respiratory rate

The data in Fig 3 show that the respiration is cyanide sensitive, 0.001 molar cyanide causes about 40 per cent inhibition In the experiment on which Fig 3 was based, the spores were in distilled water with 5 per cent KOH in the inset The HCN distills over to the KOH, and with falling cyanide concentration the respiration increases Other experiments showed that 0.01 molar cyanide completely inhibits the respiration It is probable that the respiratory mechanism depends upon the Warburg iron enzyme system

Since the activation phenomenon is reversible, and activated spores may be deactivated, the question suggests itself, whether the reversibility of the activation deactivation carries over to the respiration That the deactivation of activated spores leads to a decrease in respiration to about the level of the dormant cells is shown by Fig 4 This graph also shows that on reactivation the respiration returns to at least as high a level as on the first activation The reversibility of the activation process is shown by the effect on respiration just as it is shown on the germination

The Effect of Iodoacetate and Iodoacetamide on the Germination and Respiration

It was shown that the activation process occurs in the absence of any respiration, but it was still possible that anaerobic metabolism was essential for activation This problem was attacked by the use of monoiodoacetate and monoiodoacetamide The prior physiological use of the iodoacetamide is unknown to the author

The spores were activated in the presence of iodoacetate or iodoacetamide At the completion of activation they were separated into two samples, in one of which the cells were well washed, and in the other not The results are shown in Table IV Both of these compounds prevent germination, but iodoacetate does not prevent activation since the washed cells germinated However, iodoacetamide prevented germination even in those cells which were washed after activation This difference between the poisoning action of iodoacetate and iodoacetamide is probably due to the more rapid penetration of the latter, since it is much more lipid soluble than the iodoacetate

The inhibition of germination of neither of these compounds can be overcome by adding, at the same time the poison is added, sodium pyruvate or sodium lactate

TABLE IV

	Cells washed after activation Per cent germination	Cells not washed Per cent germination
Control	—	91 normal
Iodoacetate 0.0075	89	0
Iodoacetate 0.003	90	0 normal 51 incipient*
Iodoacetate 0.0006	88	91 normal 0 incipient
Iodoacetamide 0.005	0	0 normal 0 incipient

* Incipient germination means the formation of germ tubes a few μ long, which did not grow

All concentrations are in mols per liter

TABLE V

Time after activation	Control dormant	Control activated	Iodoacetamide added before activation	Iodoacetamide added after activation	Iodoacetate added after activation
<i>hrs</i>					
0.5-2.0	6	100	94, 6	91, 9	101, 1
2.0-3.0	7	135	92, 32	100, 26	114, 16
3.0-5.5	5	181*	74, 59†	60, 67†	86, 53‡
8.0-9.0	7	151	—	27, 82	81, 46

Temperature 24.9°C, 0.068 ml of spores, concentration of iodo compounds 0.005 molar, suspension in phosphate buffers M/60, pH 5.9

Figures in cmm of oxygen consumed per hour, bold-faced figures represent per cent inhibition

* Germination 93 per cent

† No germination

‡ No normal germination, 47 per cent incipient germination

The effect of these two iodo compounds on the respiration of the activated spores was determined at 0.005 molar concentrations. The same suspensions were in M/60 phosphate buffers at pH 5.9. The results of a typical experiment are given in Table V. The iodoacetate

and iodoacetamide do not appreciably inhibit the primary increase in respiration which follows activation but they prevent the secondary rise in respiration and the germination which accompanies it, that occur in controls. The iodoacetamide differs from the iodoacetate in its effects on the activation of the spore. Spores activated in the presence of the iodoacetamide and then washed will not germinate, but the primary increase in respiration occurs in these spores.

Methods

The *Neurospora tetrasperma* cultures were obtained from Dr. B. O. Dodge and bore his number 193 e. *N. tetrasperma* is a homothallic (bisexual) species having 4 spored asci. It grows rapidly in culture and after 10 to 15 days at 25°C produces perithecia and discharges its ascospores. The cultures were raised in Blake bottles on malt extract agar or beer wort agar. The malt extract agar consisted of 0.5 per cent Difco malt extract, 0.5 per cent glucose, and 2.0 per cent agar. The spores were removed from the Blake bottles by washing with distilled water. Care was taken to exclude as many conidia as possible. These were separated from the contaminating conidia by allowing them to settle several times through a column of water. The large difference in density soon separates them. Centrifuging in 50 per cent cane sugar also gave a good separation. The spores from 30 to 50 cultures were lumped, air dried and stored over saturated solutions of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ and its crystals (32 per cent relative humidity at 20°C) or over concentrated solutions of NH_4Cl and solid NH_4Cl (79 per cent humidity at 20°C).

The age of the spores up to about 18 days affected slightly the critical temperature of activation, but from 28 to 90 days the spores remained constant. Different spore lots varied about 2° in the position of the critical temperature, but in no other manner. The same spore lot gave surprisingly constant results, if stored at constant humidity, over several months. Except where noted all the data are on the same spore lot.

I

The spore suspensions were made up in distilled water or in solutions of 0.5 per cent malt extract, 0.5 glucose and 0.016 molar phosphate buffers at pH 6.2. The spores were heated in thin capillary tubing which had an external diameter of 1.0 to 1.2 mm. The heating was in a water bath constant to $\pm 0.03^\circ\text{C}$. The tubes were immersed in cold water at the termination of the heating. After activation the spores were spread on a cover slip, a drop of melted clear corn meal agar (or agar alone) cooled to 44°C was added, and the cover slip was sealed to a depression slide with liquid petrolatum. Three different samples of spores were used and in Fig. 1 the three curves correspond to the three spore lots.

II

The methods were identical with those above. The same spore lot was used to determine the curves at the different temperatures.

III

The spores were heated in Thunberg tubes in air (controls), in nitrogen, in carbon monoxide, or in solutions of HCN. The tubes were successively evacuated and refilled with nitrogen freed of oxygen by passing it over heated reduced copper. It is easy to obtain anaerobic conditions that will prevent germination, but if deactivation is to occur in a few hours no traces of oxygen may remain. Traces of oxygen were removed by having acid chromous chloride in separate containers in the Thunberg tubes. The carbon monoxide was generated from formic and sulfuric acids and freed of carbon dioxide by passing through KOH. Fresh KCN solutions were neutralized to phenol red. The spores were activated by heating the Thunberg tubes in a water bath at 54–55°C for 20 minutes. They were cooled, and placed on a shaking machine and shaken at such a rate that the spores in air gave 93–95 per cent germination. At various times, the tubes were opened and the spores planted in agar to determine germination. HCN and CO were first removed by evacuation. Duplicate tubes were reheated without opening to determine the possibility of reactivation.

IV

Care must be taken if the ascospores are to be obtained free of vegetative contamination (conidia, perithecia, and mycelia). The ascospores from 50 Blake bottle cultures give enough spores for 10 to 12 Warburg vessels using 0.05 ml of spores per vessel. The spores were passed through a fine mesh screen to remove perithecia and mycelia and settled several times through a tall column of water to remove the conidia. The difference in density between ascospores and conidia gives a rather good separation.

V

The amide of iodoacetamide was prepared from chloroacetamide by the method of von Braun (12). It had a melting point of 95–96°C which did not change on recrystallization from water or chloroform. The spores were activated as above, with the iodocompounds added before activation. Iodoacetate was neutralized to methyl red. After activation, part of the spores were washed free of the poison on the centrifuge, the other part was put on the shaking machine for several hours. Germination counts were made after 6–8 hours and after 24 hours. No spores that had failed to germinate in the former time germinated after 24 hours.

The respiration experiments were similar to those above. The suspensions were made in $M/60$ phosphate buffers pH 5.9. The iodoacetate was neutralized, and the concentration of iodoacetate and iodoacetamide was 0.005 molar. The volume of the spores in the experiment reported was 0.068 ml.

SUMMARY

The heat activation of *Neurospora tetrasperma* ascospores is a reversible process, since activated spores may be returned to secondary dormancy by preventing respiration, and these secondarily dormant spores may be induced to germinate by reheating. Activation of the spores brings about a large increase in respiration prior to the germination of the spores. As the spores are reversibly activated or deactivated the rate of respiration is increased or is decreased. By poisoning the cells with iodoacetamide it is possible to prevent all germination without greatly inhibiting this increase in respiration. Precisely with the beginning of germination a secondary rise in respiration occurs. The respiration of the spores is cyanide sensitive. The heat activation has a critical temperature at about 49 to 52°C, and at a constant temperature within this range, the percentage of the spores activated as plotted against the time, follows an S shaped population curve.

It is a pleasure to thank Dr. Leonor Michaelis for constant advice and stimulation and for the facilities of his laboratory. To Professor John Runnström the writer is indebted for advice and to Astrid Runnström for instruction in the technique of manometric methods.

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STUDIES IN RESPIROMETRY

IV THE USE OF A COMPARATOR SYSTEM IN REFRACTOVOLUMETRIC RESPIROMETRY

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(Accepted for publication, February 12, 1935)

A respirometer dependent upon measurement of change in volume and refractivity of a closed system has been described previously¹ and its use under various conditions indicated,^{2, 3} among which were measurement of continuous respiration (O_2 and CO_2) without chemical absorption in wet or dry systems. In these studies the CO_2 measurements for small time intervals are obviously more reliable than those of the corresponding O_2 changes. This appeared to be due largely to sudden although small variations in pressure which could not be followed readily with a barometer. The object of the present communication is to report the result of later work, where we have used a comparator system to compensate for such variations in similar studies of oxygen and carbon dioxide respiratory rates of newts.

The respiratory apparatus was the same as in former work except that the respiratory chamber containing the newts was a low flat vessel of pyrex glass (460 ml. capacity) with a portal for admission of the newts and delivery tubes for sealing (with mercury) into the respirometer in the usual manner¹⁻³. It contained 25 ml. of water the newts being about half submerged. The total gas volume in the closed system at the beginning was approximately 1.034 liters, and the temperature ranged between 30.09 and 30.31°C. As a comparator system we used a glass burette (50 ml.) in horizontal position provided at the end remote from the stop cock with a stopper through which was attached a micro burette also horizontal, containing a small drop of kerosene. A small drop of water was introduced into the body of the large burette to provide a water saturated atmosphere as in the respirometer, and small pieces of rubber tubing were placed at intervals

¹ Thompson, W. R., *J. Gen. Physiol.*, 1932-33, 16, 5

² Thompson, W. R., and Tennant, R., *J. Gen. Physiol.* 1932-33, 16, 23

³ Thompson, W. R., and Tennant, R., *J. Gen. Physiol.* 1932-33, 16, 27

about the burette as insulation so that the rate of temperature change of the comparator system approximated that of the respirometer under the conditions of use. The residual errors from failure to exactly compensate in this manner were reduced by the thermoregulation of the room as described previously¹. Readings of volume change in the comparator could thus be made simultaneously with those of the burette of the respirometer, but, in order to facilitate this, a stop-cock was provided at the remote end of the micro burette which when closed practically

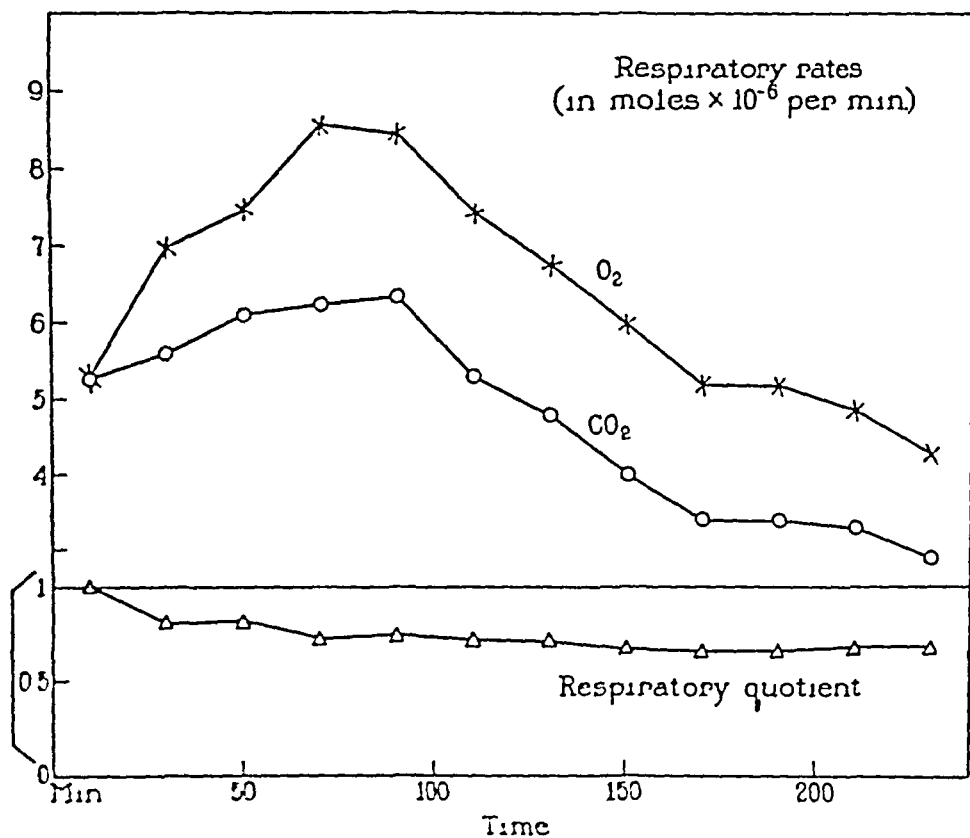


FIG 1 Continuous respiration of American newts partly immersed in water

former work,³ and the respiratory quotient (indicated on a sub graph in the same figure) is fairly stable, in spite of the fact that the changes measured are only about half as great

The temperature pressure change factor is obtained directly from the volume variation in the comparator. This gives a correction not only in an extremely simple form, but one which closely represents the condition at the time of measurement of the volume of gas in the respirometer. A possible improvement might be to have a comparator chamber sealed within one of the conduits of the respirometer with only a small portion protruding, the horizontal micro burette with kerosene bead and stop cocks

In work where relative humidities between 0 and 1 are required, long horizontal tubes partly filled with an equilibration mixture, e.g. solid salt under a saturated salt solution, may be introduced into the circulation system and into the pipe line through which fresh air is drawn at the beginning

ACTION POTENTIALS IN THE NERVOUS SYSTEM OF THE CRAYFISH*

IV INFLUENCE OF TEMPERATURE ON NERVE IMPULSES ARISING "SPONTANEOUSLY" IN ABDOMINAL GANGLIA†

By C LADD PROSSER

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(Accepted for publication, March 5, 1935)

Nerve impulses continue to be emitted from ganglia in the abdomen of a crayfish even after all nerves connected with these ganglia are cut (Prosser, 1934). A method has been described (Prosser, 1934) for the analysis of these "spontaneous" discharges into rhythmically recurring impulses in individual axons. Concentric electrodes (Adrian and Bronk, 1929) were used in these experiments, these have certain advantages over ordinary leads. In the first place, they distort the shapes of the impulses and thus make identification of impulses in terms of wave form easier, and secondly, it is possible to obtain records when the concentric needle electrodes are inserted through the epidermis under the nerve cord and the circulation is left essentially unimpaired. It is also possible to obtain records from the nerve cord when the animal is immersed in Ringer's fluid with the epidermis intact except for the small opening made by the needle. This procedure was used in the following experiments. Records were made with the aid of an amplifier and a Matthew's oscillograph.

Before immersion of the specimen the cord was cut anterior to one abdominal ganglion, and just behind the adjacent posterior ganglion and the electrodes were inserted beneath the nerve cord between the ganglia without disturbing the epidermis over them. In some of the experiments the segmental nerves from each

* Two previous papers in this series have appeared in *J Cell and Comp Physiol* 1934, 4, 185-363, and one in *J Comp Neurol*, in press.

† This investigation was made possible by a grant from the Rockefeller Foundation to Professor Hudson Hoagland, Clark University for research in physiology.

ganglion were cut, but this was found to have no influence on the results and tended to make the preparation less viable. Exclusion of afferent impulses was shown by the absence of responses when distant appendages were stimulated or when the fluid covering the animal was moved. Records were made only when the preparation was quiet. The fluid covering the preparation could be siphoned out and changed without moving either the animal or the electrodes.

When the Ringer's fluid bathing the preparation was changed for one of another temperature, an immediate alteration in the number of impulses in the commissure between the two deafferented ganglia was observed. A constant equilibrium frequency characteristic of the new temperature was usually attained in from approximately 30 to 60 seconds, the thinness of the epidermis doubtless hastening equilibration. 3 to 5 minutes were allowed to elapse before photographing the nerve impulse discharge. The experiments were started in the middle of the range of temperatures to be explored and, after exposure to several lower and higher temperatures, the preparation was returned to the initial temperature. No irreversible failure of the impulse discharge was usually noted during the time of the experiment (approximately 1 hour). The few cases that showed irreversible changes during the experiment were discarded.

The photographic records were analyzed and impulses of the same time relations grouped together exactly as described in the first paper of this series (Prosser, 1934). When the electrodes were not moved during an experiment, it was possible to follow a given type of impulse at each of several temperatures. The forms of the impulses are characteristic of the single fibers producing them (Prosser, 1934).

Analysis of records of the spontaneous discharge showed that as the temperature increases the gross frequency of discharge, *i.e.* the total number of impulses on the average for 0.1 second, increases considerably (Fig. 1). The records clearly show that two factors contribute to this, (1) the increase in number of active fibers and (2) the increase in frequency of the impulses from the individual fibers. With a rise in temperature the number of active elements increases more rapidly than does the frequency of discharge of the individual fibers. In the lower temperature range this increase in number of elements is particularly large, but above 15° (Fig. 1) the curve for number of active units falls below that for the increase in gross frequency. It might be objected that as the total number of impulses becomes greater, the analysis is less accurate, and that this may account for the difference in the two curves, but very dependable analyses of eleven records using a high-speed camera have shown the same relations, and further, as the temperature rises, there seems to be a tendency for small fibers to become active.

As was shown previously (Prosser, 1934), a single fiber in these spontaneously discharging centers shows considerable moment to moment fluctuation in its frequency. It was necessary, therefore, to take the average frequency for a number of discharges. When average intervals between approximately ten discharges were measured

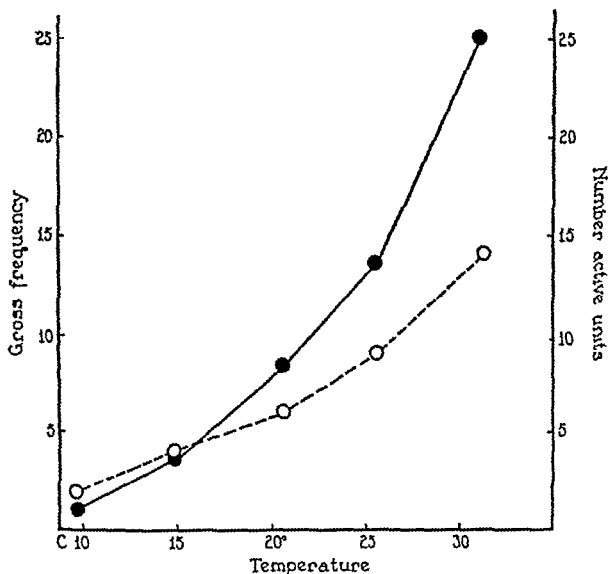


FIG 1 Plot of temperature *versus* total number of impulses per tenth second (solid line) and number of active units (broken line) in a commissure between two deafferented ganglia of a crayfish

for those fibers which could be followed at several temperatures, it was seen that some were affected more by temperature changes than were others. Curves for two fibers, one increased considerably in its frequency by rise in temperature, and the other but little, are shown in Fig 2

Curves were made by plotting the logarithms of gross frequencies (assumed to be proportional to the processes determining the frequencies) and the logarithms of number of active units (assumed to be a measure of processes determining the temperature thresholds)

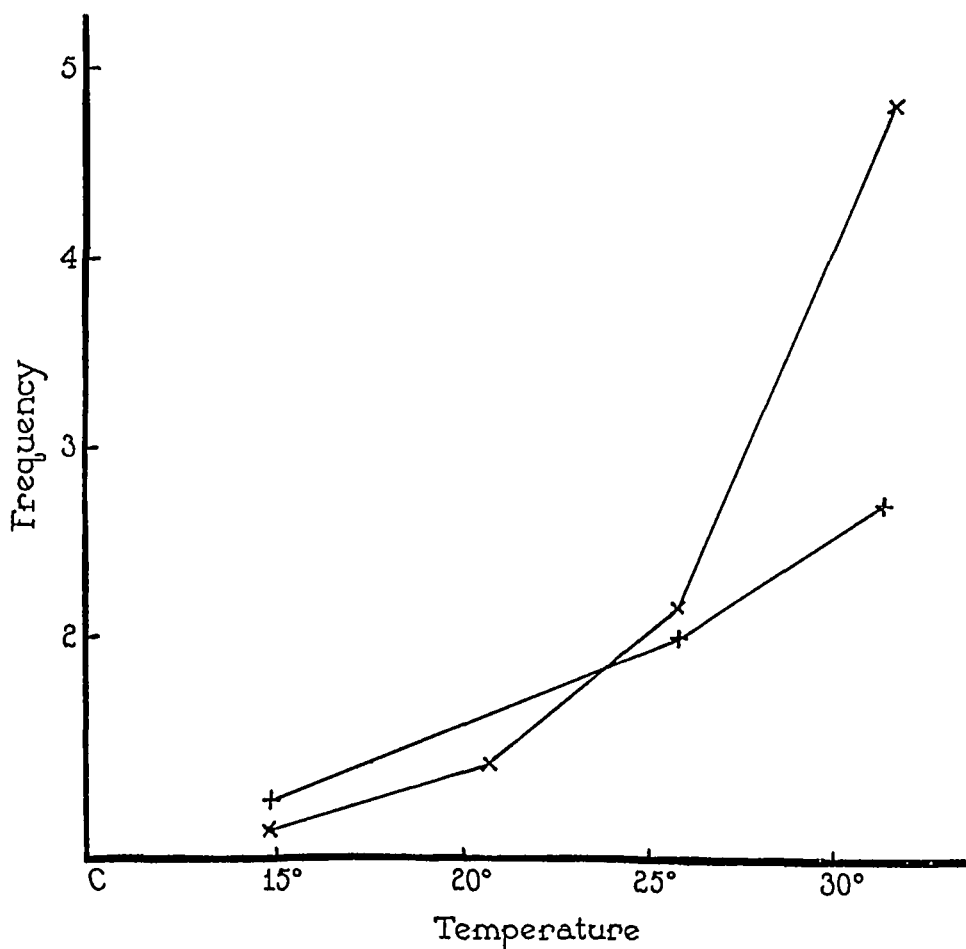


FIG 2 Change in frequency (number per 0.1 second) of two units with temperature. These are from the experiment plotted in Fig 1

against the reciprocals of the absolute temperatures. These plots test the applicability of the Arrhenius equation

$$\log \frac{k_1}{k_2} = \frac{\mu}{R} \left(\frac{1}{T_2} - \frac{1}{T_1} \right)$$

where k_1 and k_2 are frequencies at the absolute temperatures T_1 and T_2 , R is the gas constant, and μ is the temperature characteristic. The difficulties of changing the fluid without moving the electrodes made it

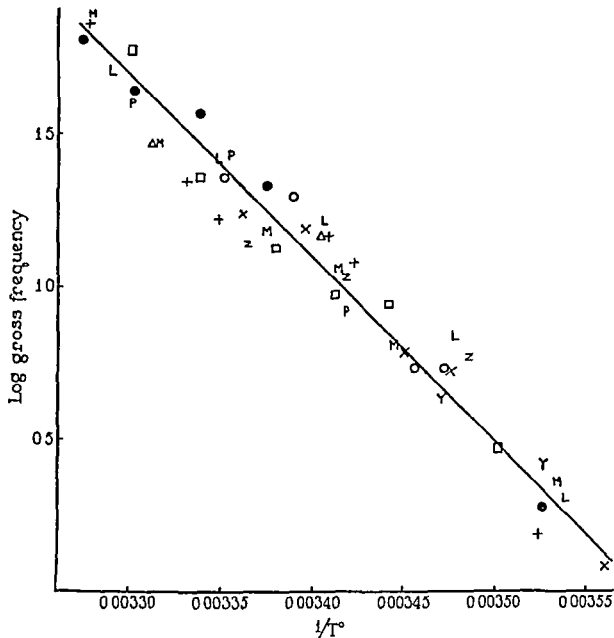


FIG 3 Plot of the gross frequency (average number per tenth second) of spontaneous discharges and temperature according to the Arrhenius equation. Each symbol designates one experiment, the same experiment in Figs 3, 4, and 5 $\mu = 27,000$ calories

impossible to obtain values at enough temperatures to give significant plots for single preparations. Consequently, the individual curves were telescoped together by superimposing them in such a way that their ordinate intercepts coincided, as shown in Figs 3 and 4. In

these plots, as in those of Fig 5, each symbol designates one experiment. The curve for gross frequency shows considerable scatter about a straight line and gives a value of $\mu = 27,000$ calories. The curve for number of active units (*i.e.*, the temperature threshold curve) is not so steep and gives a value of $\mu = 17,500$ calories.

Plots of the logarithms of the frequencies of discharge of individual units against the reciprocals of the absolute temperatures were made. It was immediately seen that the curves fell into two groups with no

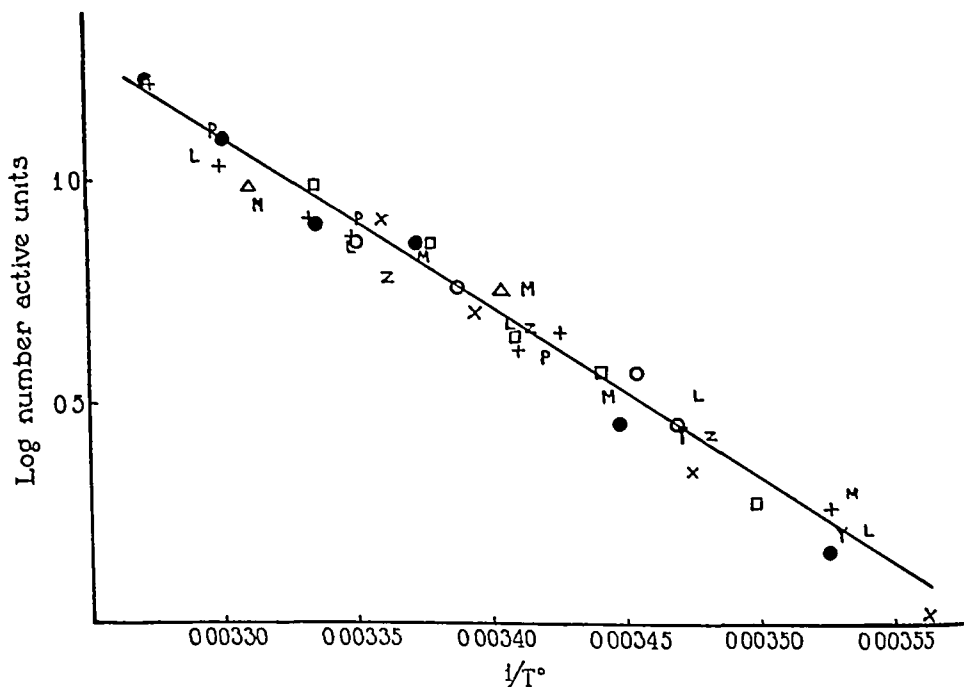


FIG 4 The change in number of active units with temperature $\mu = 17,500$ calories

appreciable overlap. When all the ordinates of the curves of these groups were telescoped together, the points give values of $\mu = 14,500$ and $7,000$ calories respectively, as shown in Fig 5.

These experiments afford an analysis of a complicated function of the central nervous system into some of its components. Each of the three processes—threshold of different units, and change of frequency in what are clearly two functional types of cells—gives a different temperature characteristic. It is of interest that the plot for the gross

tion (*cf* Crozier and Hoagland, 1929) can be applied to the present data, but it is doubtful as to whether the figures on gross frequencies of impulses would be homogeneous by this test

The only comparable measurements available concern the effect of temperature on the spontaneous discharge in the lateral-line organs in the catfish (Hoagland, 1933) The effect of temperature here was less than for the gross frequency in the crayfish ganglia and a value of $\mu = 5050$ was obtained

The fact that in the crayfish ganglia the change in number of active units with change in temperature should be of greater importance than the change in frequency of individual units is suggestive of the considerable increase in number of active units and the more limited increase in frequency of individual units with increased tension in skeletal muscle (Adrian and Bronk, 1929, Smith, 1934) This is also consistent with the original hypothesis (Prosser, 1934) that these impulses are tonic in nature

SUMMARY

Increase in temperature elicits an increase in the number of nerve impulses arising spontaneously from deafferented crayfish ganglia This alteration in gross frequency gives an apparent temperature characteristic of 27,000 calories

Changes in the number of active fibers and in the frequency of discharge of individual units account for the alterations in the gross frequency The change in number of active units gives a μ value of 17,500 calories

Individual fibers fall into two groups with respect to the effect of temperature on their frequency of discharge One of these groups exhibits a μ value of 14,500 calories and the other yields a μ value of 7,000 calories

It is a pleasure to acknowledge the helpful advice given generously by Professor Hudson Hoagland

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THE ACCELERATING EFFECT OF MANGANOUS IONS ON PHAGE ACTION*

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(Accepted for publication, March 27, 1935)

In previous papers dealing with the properties of an antistaphylococcus phage and its mode of action on a strain of *Staphylococcus aureus* (1-9) it has been shown that

1 In a mixture of phage and growing bacteria, phage in or on the cells is in equilibrium with the phage free in the medium

2 Phage formation is intimately related to bacterial growth and as far as can be determined does not occur in its absence. The rate of phage production is considerably greater than the rate of bacterial reproduction, as a consequence the ratio of phage to bacteria is constantly increasing. Upon accumulation of a certain high ratio of phage to bacteria (lytic threshold) lytic destruction of bacteria begins and proceeds rapidly to completion.

3 Phage can be completely inactivated by high concentrations of HgCl_2 and subsequently can be reactivated by removal of the Hg^{++} ions. Similarly inactivation with KCN can be reversed by conversion of the CN^- into $\text{Ag}(\text{CN})_2^-$.

None of the experimental evidence so far elicited indicates clearly the nature of phage, it may be animate or inanimate—either concept will fit the few known facts. Additional data about phage and its mode of action upon bacteria constitute an essential requirement before any serious attempt to define its nature can be made.

To continue the study of one particular phage and bacterium, the antistaphylococcus phage and *Staphylococcus aureus* above mentioned, systematic experiments on the effect of electrolytes upon the phage-bacterium reaction were undertaken. It was found, as would be

* Supported by Grant in Aid from Eli Lilly and Company

anticipated, that a variety of electrolytes in appropriate concentrations served to inhibit the reaction. Of greater interest, however, was the acceleration noted in some instances, particularly in the case of Mn^{++} salts. The Mn^{++} ion in extremely low concentrations lessens the time required for lysis to begin in a given mixture of phage and bacteria, diminishes the final yield of phage obtained, and shifts the equilibrium between intracellular and extracellular phage in favor of the latter fraction.

The Acceleration Effect

Cultures of *Staphylococcus aureus* were grown in infusion broth containing 0.00016 molar $MnCl_2$ at $36^\circ C$. When $[B]^1 = 1 \times 10^8$ 1.0 ml of the suspension was added to 4.0 ml amounts of various concentrations of phage containing 0.00016 molar $MnCl_2$. These tubes were shaken in the $36^\circ C$ water bath together with identical controls containing no manganese. The results are recorded in Table I. For any given phage concentration the manganese-containing cultures lysed 0.5 hour ahead of the controls. To determine whether this result was due to the anion or cation, experiments were run with equivalent solutions of NaCl and $MnSO_4$. There was no accelerating action due to NaCl in the concentration used while the $MnSO_4$ produced the same effect as the $MnCl_2$. The action was therefore attributed to the Mn^{++} ion.

Analysis of the Mn^{++} Effect

In considering the mechanism underlying the accelerating effect of Mn^{++} ions three general possibilities suggested themselves, first, that the ion stimulates bacterial growth, increasing the rate of phage production and hastening attainment of the lytic threshold, second, that phage formation is somehow enhanced independently of bacterial growth, third, that the Mn^{++} ion reduces the lytic threshold and renders the cells more susceptible to phage action.

In order to test out the first of these hypotheses, that bacterial growth stimulation primarily was involved, a series of experiments was run following in each case the bacterial growth curve in phage-bacteria mixtures and in similar mixtures to which had been added manganese.

¹ $[P]$ = phage concentration in activity units/ml $[B]$ = concentration of bacteria or staphylococci/ml P U = phage activity unit

TABLE I
Acceleration of Phage Action by Mn^{++}

Initial phage concentration	1×10^8 P U /ml	1×10^7 P U /ml	1×10^6 P U /ml	1×10^5 P U /ml
Initial bacterial concentration	2.5×10^7 B/ml	2.5×10^7 B/ml	2.5×10^7 B/ml	2.5×10^7 B/ml
Time of onset of lysis with Mn^{++}	0.8	1.5	2.15	2.9
Time of onset of lysis without Mn^{++}	1.3	2.0	2.6	3.4

Temperature = 36°C pH = 7.2

For the Mn^{++} series the bacteria were grown in broth containing 0.00016 M $MnCl_2$.

The phage dilutions used contained the same concentration of $MnCl_2$.

The growth curves were found to be identical as is indicated in Fig 1, a record of one experiment

To test for the possible action of Mn^{++} on the phage-forming mechanism, mixtures of phage and growing bacteria with and without manganese were incubated at $36^{\circ}C$ and samples were taken at intervals for titration of phage. Employing the procedures outlined below for quantitative determination of phage and bacteria, it was found that the rate of phage formation was not increased by manganese

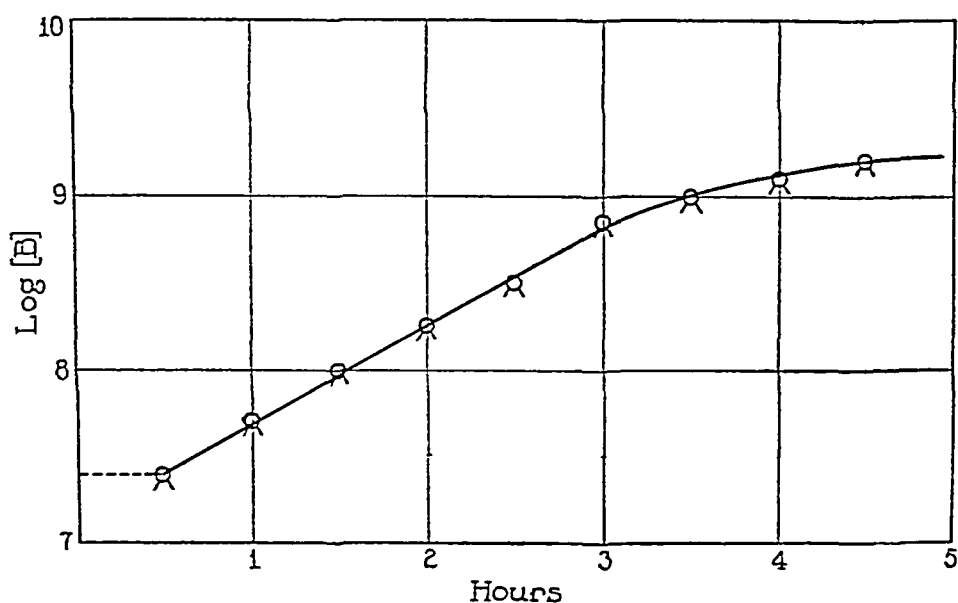


FIG 1 Bacterial growth curves with and without $MnCl_2$ as measured by turbidity method (see text) $36^{\circ}C$ $pH = 7.2$ \bigcirc = initial [phage] = 1×10^4 [bacteria] = 2.5×10^7 , $MnCl_2$ 0.00016 M \bigcirc = initial [phage] = 1×10^4 [bacteria] = 2.5×10^7 , no manganese

However, there was a decided difference in the distribution of phage between the medium and the cells. Manganese increases the extra-cellular phage fraction to about four times the ordinary free phage concentration found in the absence of manganese. This is shown in Fig 2. Distribution of phage evidently is of the simple type previously described (4) both in the presence of and in the absence of manganese. The average value of the partition coefficient K calculated from the equation $K = \frac{Pb/B \cdot 25 \times 10^{10}}{P_E}$ (where Pb = phage in bac-

teria/ml, B = bacteria/ml, and P_E = phage free in medium/ml) was 15×10^3 in the absence of manganese. With manganese present the value for a number of experiments was 4×10^3 .

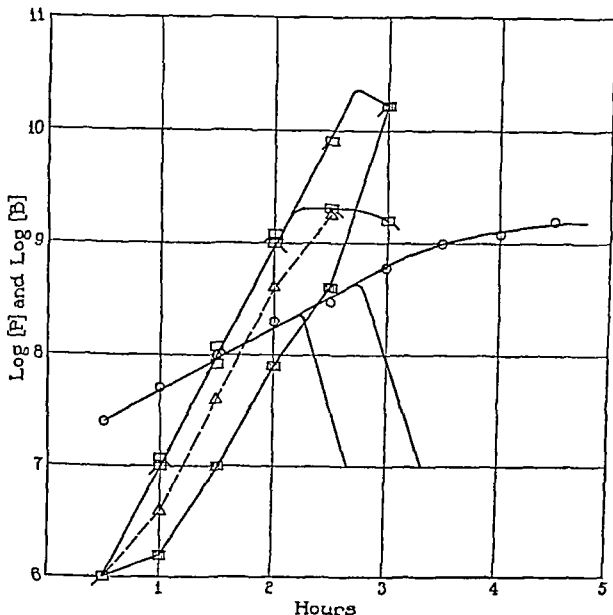


FIG. 2. The distribution of phage between bacteria and medium during growth and phage production in plain broth and in $MnCl_2$ broth. 36 C, pH = 7.2. \circ = bacterial growth curve, \square = total phage/ml in absence of Mn^{++} , \blacksquare = extracellular phage/ml in absence of Mn^{++} , \square = total phage/ml in presence of Mn^{++} , \triangle = extracellular phage/ml in presence of Mn^{++} .

It is clear from Fig. 2 that lysis requires a lower ratio of phage to bacteria in the case of the manganese mixtures. As determined in current experiments, the usual ratio of phage to bacteria at the time of

lysis in the absence of manganese is 54 phage units/cell In the presence of manganese this is reduced to a figure of about 12 (average of several experiments)

TABLE II
The Effect of MnCl₂ on the Lytic Threshold

Standard phage 1 × 10 ¹⁰ P U /ml	8 0	7 0	6 0	5 0	4 0	3 0	2 0
Broth, ml	1 0	2 0	3 0	4 0	5 0	6 0	7 0
Bacteria (8 × 10 ⁸ /ml)	1 0	1 0	1 0	1 0	1 0	1 0	1 0

Time	Lytic curves followed by means of turbidity measurements <i>B</i> = Cells × 10 ⁷ /ml													
	[B]	[B]	[B]	[B]	[B]	[B]	[B]	[B]	[B]	[B]	[B]	[B]	[B]	[B]
	With Mn	Without Mn	With Mn	Without Mn	With Mn	Without Mn	With Mn	Without Mn	With Mn	Without Mn	With Mn	Without Mn	With Mn	Without Mn
<i>hrs</i>														
0 2	8	8	8	8	8	8	8	8	8	8	8	8	8	8
0 4	6	6	6	6	6	8	8	8	8	8	8	8	8	8
0 6	3	4	4	4	4	10	6	10	6	10	8	8	10	8
0 8	0	0	0	0	0	10	4	12	4	10	6	10	12	10
1 0						6	0	12	0	12	4	12	10	12
1 2						4		12		12	0	12	8	12
1 4						0		8		12		12	6	14
1 6								4		10		10	0	12
1 8								0		6		8		10
2 0										0		6		8
2 2												0		4
2 4														0

Determination of critical phage/bacteria ratio for lysis in absence of bacterial growth

Staphylococcus-phage mixtures containing (a) 0 00016 molal MnCl₂, (b) no MnCl₂

[Bacteria] constant and [phage] varying Young bacterial cultures used and growth inhibited by preliminary icing Tubes transferred to 36°C bath and turbidity measurements made every 0 2 hour The critical phage/bacteria ratio for lysis with Mn⁺⁺ present = 38 and with no Mn⁺⁺ = 88

If the lytic threshold in growing mixtures is lowered by manganese it would be expected that similar results could be obtained with static (non-growing) mixtures of phage and bacteria To test this point

bacteria were grown in the presence of manganese and were then iced to inhibit growth. A unit volume of bacteria was added to varying concentrations of phage, the mixtures were iced to avoid bacterial growth and phage formation during the time required for establishment of equilibrium between intracellular and extracellular phage, and they were then shaken in the 36°C water bath. Turbidity measurements were taken every 0.1 hour. Table II shows the results obtained in the presence and in the absence of manganese. Evidently the lytic threshold in static mixtures of phage and bacteria is lowered as in the case of the actively growing mixtures although in the absence of growth higher phage/bacteria ratios are required for lysis.

That the accelerating effect of manganese is due to depression of the lytic threshold is evidenced also by the reduction in final titre of phage when Mn^{++} is present. Experimentally, for any given initial phage concentration the end titre is uniformly about 1/10th of that developed in the absence of manganese. This would be anticipated if a lower ratio of phage to bacteria is required to institute lysis. (See Fig. 2.)

Methods

A. Materials and Titration Technique—The broth used in our work was beef infusion containing in each liter 10 gm. Fairchild's peptone and 5 gm. NaCl. The hydrogen ion concentration was adjusted to between pH 7.2 and pH 7.4.

The stock manganese solutions contained 0.08 molar $MnCl_2$ or $MnSO_4$ and were diluted in physiological saline solution to 0.0016 molar strength before each run. Final 1:10 dilutions were made in broth so that the latter contained 0.00016 molar $MnCl_2$ or $MnSO_4$.

18 hour cultures of *Staphylococcus aureus* grown in Blake flasks were suspended in saline solution for each day's experiments. In order to have a uniform cell suspension the bacteria were seeded into manganese broth and plain broth and were grown at 36°C until the bacterial concentration was sufficient for use. Measurements of bacterial concentrations were done by a simple turbidity comparison method as described by Krueger (1).

The bacteriophage was the antistaphylococcus phage used in previous experimental work (1-9). The standard phage solution contained 1×10^{10} activity units/ml.

Quantitative determinations of phage were routinely done by the method previously described by Krueger (1). In this method varying dilutions of unknowns and of standard phage are mixed with a unit concentration of susceptible bacterial cells. The time of lysis, i.e. the length of time required to reduce the

growing suspension to a particular turbidity end-point, is determined under controlled conditions. The smaller the amount of phage present the longer it takes for the suspension to lyse and the initial phage concentration of an unknown is read directly from a graph in which the time of lysis of the standard controls is plotted against the logarithms of phage units present. The phage unit is an arbitrary one and represents the minimum quantity producing complete clearing of 1.25×10^8 cells in 5.0 ml pH 7.6 broth at 36°C.

For determining phage free in solution in mixtures of phage and bacteria, the bacterial cells were centrifuged down and the supernatants diluted and titrated as mentioned above. In the presence of Mn^{++} ions such dilutions do not yield true titration values because of the accelerating action of manganese on the titration mixture, the values are always too large. For this reason it was found necessary to add some substance which would inhibit the manganese effect on the titration set-up, 0.00016 Na_2SiO_3 in broth was found satisfactory for this purpose. In practice manganese-containing phage unknowns were diluted for titration in the silicate broth. Manganese-containing controls were diluted with plain broth and with silicate broth to make sure that the manganese effect was successfully blocked by the silicate.

To determine total phage/ml, i.e. intracellular phage + extracellular phage, various dilutions of the phage-bacteria mixtures were titrated just as in the case of the supernatants. It was found that Na_2SiO_3 would not inhibit the manganese effect in such mixtures and consequently they titrated too high. It was therefore necessary to include control sets with each titration consisting of a number of bacterial concentrations representing points on the logarithmic growth curve to which had been added varying amounts of phage. The mixtures were iced to allow the intracellular and extracellular phage fractions to come to equilibrium and were then diluted. The usual aliquot of bacterial suspension for phage titration was added to each tube and the time of lysis was determined in the routine way. By means of this additional series of controls it was possible to determine the amount of phage in the given manganese-containing mixture providing the concentration of bacteria originally present was known.

B The Accelerating Effect of Mn^{++} on Phage Action—Phage dilutions in plain broth and in manganese broth were prepared using 4.0 ml amounts of 1×10^8 , 1×10^7 , 1×10^6 , 1×10^5 , and 1×10^4 phage units/ml. To the plain broth-phage dilutions were added 1.0 ml of a growing staphylococcus suspension containing 12.5×10^7 bacteria/ml. The manganese-phage mixtures were similarly seeded employing a staphylococcus culture growing in manganese broth. All the tubes were shaken in the 36°C water bath and turbidity measurements were made at intervals as described in a previous paper (1).

C The Effect of Manganese on Phage Production and on Phage Distribution—To 35 ml of broth there was added 5.0 ml of phage solution containing the proper concentration of phage units/ml and 10.0 ml of a broth culture of growing staphylococci containing 12.5×10^7 bacteria/ml. An identical set-up was prepared at the same time using manganese broth, phage diluted in manganese broth, and

bacteria growing in manganese broth. Samples for the determination of total phage/ml and extracellular phage/ml were taken at half hour intervals. The bacterial growth curves and lytic curves were followed by the turbidity method mentioned above. All dilutions for phage titration were kept in ice water until completion of the experiment when all were titrated at once. An experiment of this sort is shown in Fig 2. In this particular case 50 ml of phage suspension containing 1×10^7 phage units/ml was added to 350 ml of broth together with 100 ml of bacterial suspension. The final phage concentration therefore was 1×10^5 phage units/ml.

D Determination of Lytic Threshold—The lytic threshold in growing mixtures was determined from the accumulated experimental data of the distribution experiments. For the threshold in the absence of bacterial growth, bacteria were grown in plain broth and manganese broth. When $[B] = 8 \times 10^8$ the suspension was iced for 15 minutes to stop growth. Mixtures with various dilutions of plain phage and similar dilutions of phage in manganese broth were then made and the tubes were iced 0.5 hour. The mixtures were transferred to the 36°C water bath and turbidity measurements were made every 0.1 hour.

DISCUSSION

It has long been known that dilute solutions of certain electrolytes will stimulate the action of enzymes. Falk (10) showed that dilute solutions of $MnSO_4$, $MnCl_2$, $MgSO_4$, $CaCl_2$, and $BaCl_2$ increased the activity of castor bean lipase toward ethyl butyrate. Calcium and magnesium salts accelerate tryptic digestion and the neutral salts of strong monobasic acids enhance the activity of salivary amylase (11). Aluminum sulfate and monophosphates in dilute solution have been found to stimulate enzyme activity (12), potassium bromate in low concentrations stimulates the digestion of casein by trypsin (13). In some cases the mechanism of the stimulating action of electrolytes on enzymes has been worked out. For example, the chlorides of the alkaline earths stimulate the hydrolytic action of pancreatic lipase and Pekelharing (14) has shown that the action probably consists in saponification of the fatty acids thereby removing them from the system and allowing hydrolysis to proceed. Not only is it possible to stimulate the activity of enzymes with salts but their presence is also capable of increasing enzyme production by microorganisms (15).

The experiments performed in connection with the accelerating effect of manganous salts on phage action have shown that it is not due to a stimulation of bacterial growth nor to an enhancement of

phage formation There is a clear-cut lowering of the lytic threshold and also a change in the distribution of phage between the bacterial cell and its environment. The Mn^{++} ion increases the extracellular fraction at the expense of the phage fraction associated with the cell. In previous work it was not possible to determine which of the two

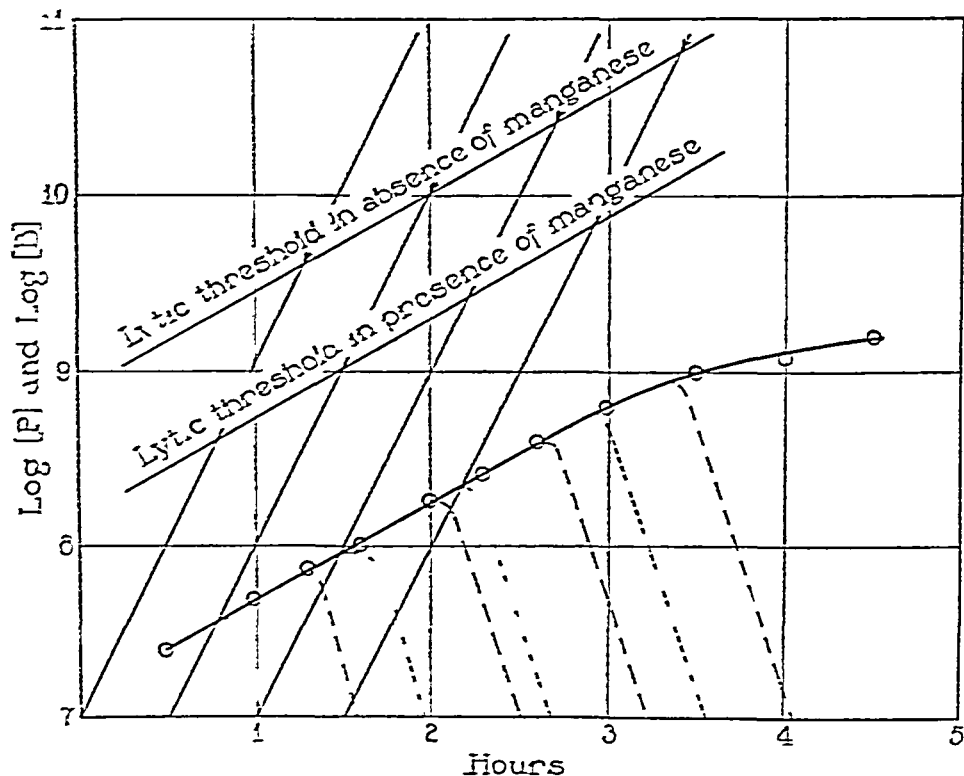


FIG 3 Graphic representation of bacterial growth, phage production, and lytic ratios with and without $MnCl_2$. Originating in the bacterial growth curve (O) are the curves of bacterial lysis (-----) with $MnCl_2$ and adjacent broken lines (-----) the identical mixtures without $MnCl_2$. Above are two lines paralleling the logarithmic phase of the growth curve, both are crossed by a series of steep curves representing phage formation for various initial phage concentrations. The intercepts of these latter lines with the two parallel lines indicate attainment of the lytic phage/bacteria ratios requisite for lysis. At corresponding time intervals on the curve for bacterial growth the curves of lytic destruction of bacteria begin. With Mn^{++} present the total phage concentrations at lysis are constantly one-tenth of those prevailing in its absence, predicting that for any given initial [phage] in a growing mixture of phage and bacteria, the presence of Mn^{++} will reduce the end titre to one-tenth that of a control mixture.

fractions, intracellular or extracellular phage, is requisite for lysis. The conclusion was reached that the significant condition for the occurrence of lysis is attainment of either a particular concentration of phage inside each bacterium or a certain concentration of phage in each ml. of surrounding solution, these two quantities are always in constant ratio to each other and it was not possible to invoke either fraction alone under equilibrium conditions.

In the manganese mixtures lysis occurs when total phage/bacterium = 12 as compared with a total phage to bacteria ratio of 54 in controls. In the present experiments the intracellular phage/bacteria ratios and extracellular phage/bacteria ratios at lysis for various mixtures of phage and bacteria were not constant. The essential ratio for initiation of lysis seems to be total phage/bacteria, that is, intracellular + extracellular phage per bacterium.

The essential features of manganese acceleration are graphically shown in Fig. 3. In this plot the bacterial growth rate is seen to be identical with and without manganese. Similarly the rate of phage production is not changed by Mn^{++} nor is the rate of bacterial destruction altered once lysis begins. The reduced threshold for lysis is represented by a line paralleling the logarithmic portion of the growth curve. Intercepts of the phage curves with this line indicate points at which the critical ratio of phage to bacteria requisite for lysis is attained and the bacterial lytic curves take their origin from the growth curve at these points. In the absence of manganese, bacterial growth and phage production continue until about ten times more phage has been produced, when lysis ensues. The lytic threshold in the absence of manganese is represented by a line paralleling the growth curve and connecting points on the phage production curves which represent the critical ratios of phage to bacteria essential for lysis. From this plot it is apparent that for any given initial concentration of phage the end titre in the manganese containing mixture after lysis will be one tenth that developed in the control.

SUMMARY AND CONCLUSIONS

Dilute solutions of $MnCl_2$ or $MnSO_4$ accelerate the lytic effect of phage upon susceptible staphylococci. Under the conditions of our

experiments the manganese-containing mixtures lysed regularly 0.5 hour sooner than the controls

The effect is shown to be due to a lowering of the lytic threshold, i.e. the quantity of phage/bacterium requisite for lysis, Mn^{++} reduces the ratio from 54 to about 12. In the presence of Mn^{++} phage distribution is altered and in growing phage-bacteria mixtures the extracellular phage concentration is increased by manganese to approximately 4 times that occurring in the absence of manganese. There appears to be no enhancement of phage formation nor any effect on the rate of bacterial growth. As would be anticipated, for any given initial phage concentration the end titre after completion of lysis is less in the presence of manganese than in its absence. This is due to the reduced lytic threshold produced by Mn^{++} , there consequently being less phage needed to bring about lytic destruction of the bacteria.

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ELECTROPHORESIS OF STEROLS

III FURTHER INVESTIGATIONS OF CHOLESTEROL SURFACES

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It has been known for many years (1) that cholesterol crystallized from solutions containing water (*e g*, ether or 95 per cent alcohol) takes up one molecule of water of crystallization. This water is readily lost at 100° or on drying *in vacuo*. Bomer (2) reported that crystals formed under such conditions belong to the triclinic system. Although the crystal has the same appearance after drying, Kofler and Kofler (3) characterize it then as a pseudomorph. On the other hand, cholesterol which has been crystallized from water free solvents has a needle like appearance and belongs to the rhombic system (3). Crystals formed from sublimation are right angled leaflets or columns, which also are rhombic (3).

In several recent communications (4, 5) the writer reported on the electrophoretic behavior of powdered cholesterol crystals which had been crystallized from alcohol following the method of Anderson (6). It seemed of interest to investigate the peculiarities in the behavior of cholesterol, if any, which are conferred upon it by the original solvent and the resultant crystal system. Sols described in our previous work were prepared by grinding cholesterol with ice (1:1) at -10°C. At that time, this was the only method by which we were able to obtain particles small enough to stay in suspension during the measurements. Since then, it has been found possible to form crystals of sufficiently small size by rapid crystallization. An opportunity to investigate the effect of the grinding process on the crystal surface thus presented itself.

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The present communication is an attempt to throw light on (1) the influence of the solvent, (2) the influence of the crystal system, and (3) the effect of grinding in contact with ice

EXPERIMENTAL RESULTS

Cholesterol from spinal cords of cattle was purified by the method of Anderson (6) and crystallized from 95 per cent alcohol (preparation A) Another sample from the same source¹ was purified independently by the same method This was then recrystallized three times from absolute alcohol and twice from freshly distilled acetone (preparation B) Preparation A, *m p* 148.5–149° (cor), had the appearance of leaflets or plates while preparation B, *m p* 148.8–149.4° (cor), was needle-shaped Samples from these preparations were recrystallized from these solvents and dried at different temperatures for varying times, under vacuum These secondary recrystallizations were carried out with the container in an ice bath while stirring rapidly to produce very small crystals After drying, these crystals were suspended in the buffers by a quick shake of the test tube Such suspensions, although not stable, were satisfactory for use during the short time required to make a measurement

The same microelectrophoresis technique was used as before (4, 7) Isoelectric points were determined in HCl solutions and mobility measurements were made at pH 5.8 in sodium acetate-acetic acid buffers ($\mu = 1/150$) All mobility values are corrected to 25°C Abramson and others (8) have shown that electrophoretic mobility is independent of size and shape within wide limits This was also found to be true in these experiments The presence of excessively large particles seemed to produce irregularities, possibly by causing turbulent motion, such particles were carefully removed before introducing the solution into the cell As may be seen from Table I, the unground material had the same isoelectric point as the ground cholesterol (4) and ergosterol (5) but its mobility at pH 5.8 was over twice that of the ground material (Table II) Each mobility value given in this report represents the average of at least twenty measurements, made as objectively as possible It will also be noted that no significant differ-

¹ Eastman Kodak Company

ences can be seen between the behavior of crystals from alcohol or acetone or under different conditions of drying. It was found that cholesterol which had been subjected to long drying at high temperatures frequently exhibited irregular mobilities. Since Kofler and Kofler (3) report that crystals sublime at 105–115°C under a pressure of 11 mm of mercury, this may be a factor involved in these deviations.

To exclude any possibility that these results might be due to the influence of slight traces of adsorbed solvents, several grams of cholesterol were melted in a pyrex beaker in an oil bath at 149°C and immediately cooled. After cooling, the surface of the crystalline mass was

TABLE I

Comparison of Electrophoretic Data Obtained with Unground Suspensions Crystallized from Different Solvents

Preparation	Crystallizing agent	Vacuum drying		pH of isoelectric point (in HCl)	Mobility at pH 5.8 μ /sec/volt/cm
		Time	Temperature		
		hrs	C		
A	Alcohol	24	80	3.0	3.79
A	Alcohol	15	90	—	3.46
B	Acetone	24	25	3.0	3.72
B	Acetone	36	100	—	4.36*
B	Acetone	15	90	—	3.50
B	Acetone	Melted at 149 °		2.9	3.77

* This experiment varied unaccountably from the rest. The higher mobilities may have been caused by the severe drying treatment.

† See text for details.

removed and material from the center of the block suspended in the buffer. No essential change in mobility was obtained (*cf* Table I).

In general, the data on unground cholesterol are more divergent than those obtained with ground material. It should be emphasized that traces of gaseous impurities, excessive heat, or long contact with organic surfaces under vacuum (*e.g.*, rubber), may yield aberrant results but if proper precautions were taken, satisfactory agreement was obtained.

If unground cholesterol was heated after suspension in water, mobilities were highly irregular. As a rule, the mobility increased and the isoelectric point dropped in pH. This agrees with our previous data.

(4) Material allowed to stand after suspension in water (without heating) also became variable in behavior, possibly due to adsorption of substances from the glass. No reliable data were obtained for these two cases

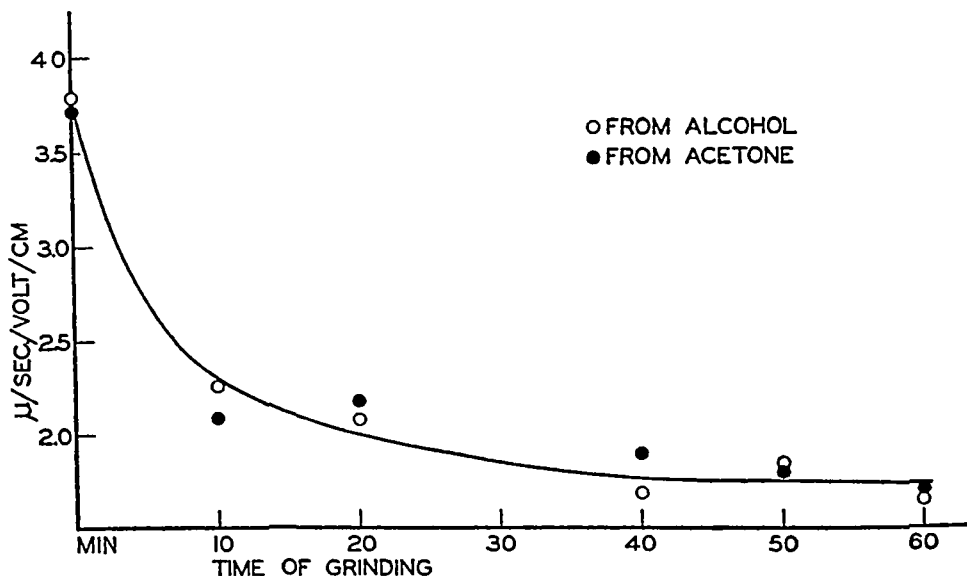


FIG 1 Changes in electrophoretic mobility produced by grinding cholesterol, crystallized from various solvents, with ice. All measurements were made at 25°C in buffers at pH 5.8

TABLE II

Comparison of Electrophoretic Data Obtained with Cholesterol Which Had Been Ground with Ice at -10°C for 1 Hour

Preparation	Crystallizing agent	Condition	pH of isoelectric point (in HCl)	Mobility at pH 5.8 μ/sec/volt/cm
A	Alcohol	Undried	3.0*	1.55*
A	Alcohol	Undried	2.9	1.65
B	Acetone	Undried	2.8	1.71
A	Alcohol	Dried†	3.1	3.55
B	Acetone	Dried†	3.1	3.22

* Previous data (4)

† Dried 15 hours *in vacuo* at 25°C

Following our previous technique (4, 5), cholesterol from both these preparations was ground with ice in an agate mortar. Grinding was done by hand, at -10°C, at as constant a rate as possible. Fig. 1

shows the changes in electrophoretic mobility (in $\mu/\text{sec}/\text{volt}/\text{cm}$) at pH 5.8 of cholesterol ground for varying lengths of time. As shown, there seems to be no significant difference between cholesterol crystallized from either solvent. After 50 minutes grinding a constant mobility is reached. Our previous measurements were made with material which had been ground for 1 hour. These and the present results (on material ground 1 hour) are compared in Table II. The term "undried" means that the material was not separated from the ice after grinding but suspended directly in the water (as before (4)) used to dilute the buffer to the proper ionic strength (1/150).

Samples were also taken from the fine powder of ice and cholesterol (ground for a full hour) and vacuum dried for 18 hours at 25°C . The results in Table II show that mobility values revert toward those obtained with unground material (Table I). Attempts were made to grind at room temperature without ice but the crystals always smeared into a single mass.

DISCUSSION

It should be pointed out that these results do not invalidate our previous data (4) where it was shown that sols prepared by the different methods in the literature show great variations in electrophoretic mobility. These fluctuations are probably due to adsorption of solvent and to heat.

The present data indicate that there is no significant difference between the behavior of the needle shaped cholesterol crystals from acetone solutions, the platelets from alcohol, or the columnar types produced by crystallization from the melted state. There is, however, a difference in mobility produced by grinding in ice. This phenomenon is, at present, difficult to account for satisfactorily. A chemical change seems excluded because of the reversibility of the reaction. A decrease in κr below the critical point ($\kappa r < 10$) would produce smaller mobilities (9, 10), but since reversal takes place on drying, this explanation would not be valid, unless drying causes a change in the effective radius. No visible increase in aggregation could be detected. Furthermore, in the ionic strength used, no mobility differences could be noticed between aggregates of very small particles of ground cholesterol in the same focus, this indicates that κr was at least > 30 .

Adam (11) gives a good discussion of amorphous surfaces produced on crystals by polishing and grinding. It is possible that the grinding has altered the orientation of the surface layers but it is hard to account by this theory for the return to the original speed on drying. The presence of films of gases on the dried surfaces which would be driven off by grinding might be advanced to account for the altered behavior but it is difficult to explain, in this manner, the constancy of the isoelectric point at *ca* pH 3.

The most plausible explanation seems to be a change in wetting such as that reported by Devaux (12) and later by Pockels (13). Devaux found that when various substances are melted on water and allowed to solidify, the under surface is wetted by water while the top surface is not. Pockels repeated this experiment with tallow, trimyristin, paraffin, wax, colophony, shellac, stearic acid, and palmitic acid. She confirmed Devaux' work except in the case of paraffin. If these solid cakes of material were inverted for several days so that the layer formerly in contact with water was now exposed to air and *vice versa*, the side now under water became wettable whereas the surface which was formerly wettable now resisted wetting. This reversal was not complete in all cases but could always be noted to a certain extent. Glass, porcelain, and platinum likewise showed this reversibility of wetting. She also discovered that glass and platinum could be made wettable by rubbing in contact with water.

Fairbrother and Varley (14) have shown that sintered glass powder becomes hydrated on standing in water for several days. Concomitant with this change, they noticed a decrease in the ζ -potential. Briggs (15) also reports these phenomena in the case of cellulose in contact with water. He mentions that "this decrease in ζ -potential approaches a limit with time and after a week the relative change per day is small. However, if the cellulose is placed in a ball-mill and beaten for a while, the ζ -potential again decreases and the decrease seems proportional to the degree of beating." This may mean that results obtained with the ground cholesterol suspensions represent the behavior of the sterol when in a steady state with its continuous phase. Grinding the cholesterol in intimate contact with ice may accelerate the rate of attainment of this steady state. It is possible that an increased adsorption of water molecules may lessen the number of

active places at the interface available for adsorption of ions and hence decrease the net charge per unit area. The ratio of positive and negative ions on the surface would only be a function of pH and hence the isoelectric point would remain unchanged.

SUMMARY

1 No significant differences were noted between the electrophoretic mobilities of unground cholesterol crystallized from alcohol, acetone, or the melted state.

2 On grinding with ice at -10°C the mobility drops to less than half (at pH 5.8) that observed in the unground state. This equilibrium condition is reached after 50 minutes grinding. Cholesterol crystals from alcohol or acetone behave identically throughout the course of this change.

3 When cholesterol crystals which have been ground with ice are dried *in vacuo* at room temperature they revert to the mobility of unground cholesterol. The cause of this phenomenon is discussed.

4 Both ground and unground cholesterol have an isoelectric point near pH 3.0, even after crystallization from the melted state.

It is a pleasure to acknowledge the interest and help given by Professor R. A. Gortner during these investigations. The writer also wishes to thank Dr. E. W. Flosdorf for drying certain samples of cholesterol. Thanks are due to Drs. H. A. Abramson and H. B. Bull for certain suggestions embodied in the discussion.

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THE MOLECULAR WEIGHT AND ISOELECTRIC POINT OF THYROGLOBULIN

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Although thyroglobulin is the principal protein elaborated in the thyroid gland, and is possibly the actual thyroid hormone, it has not been subjected to study by the more recent physical and chemical methods available for protein research. This has been due in part to the greater interest aroused by its crystalline degradation product, thyroxine, and in part to the classical methods of preparation which failed to remove entirely impurities with undesirable properties or resulted in a more or less denatured product. It is now possible, however, to prepare large amounts of thyroglobulin, free from denatured material and nucleoprotein (1). The thyroglobulin so obtained shows the properties of a pseudoglobulin¹ and a detailed study of it has been undertaken and will form the subject of a series of communications.

The present paper deals with the molecular weight and isoelectric point of thyroglobulin, two fundamental properties which are of interest both from the standpoint of protein chemistry and from that of hormone chemistry and physiology.

EXPERIMENTAL

1 Thyroglobulin Preparations—The thyroglobulin preparations used were (a) a dialyzed portion of lot J 13 B₂ described in (1) (b) a dialyzed fresh sample

* John Simon Guggenheim Fellow, autumn of 1934

¹ 'Pseudoglobulin' is here used in the sense of a globulin soluble in water. Thyroglobulin activity has recently been stated to be in a euglobulin fraction of high molecular weight (2).

² For a preliminary note of Heidelberger, M., and Svedberg, T. *Science* 1934, 80, 414.

isolated according to (1) from hog thyroids obtained in Stockholm through the courtesy of Prof E Hammarsten and Dr E Jorpes, and (c) a dialyzed sample of human thyroglobulin prepared according to (1) from a normal thyroid made available through the kindness of Dr Wm Barclay Parsons (a) Contained 0.53 per cent of iodine and 0.02 per cent of phosphorus, (b) showed 0.58 per cent of iodine and 0.02 per cent of phosphorus, and (c) contained 0.70 per cent of iodine and 0.06 per cent of phosphorus (a) Also showed 1.1 per cent of serum proteins (quantitative analyses by the precipitin method by Mr H E Stokinger)

2 *Specific Volume of Hog Thyroglobulin*—A 10 cc pycnometer was used with a 2 per cent solution of thyroglobulin and a 20 cc pycnometer with a 1 per cent solution. Estimation of the thyroglobulin content of the solutions by the micro-Kjeldahl method (thyroglobulin contains 15.8 per cent N (1)) resulted in a value of 0.71 for the specific volume, while 0.72 was found when the protein content of the solution was estimated from the dry residue (const wt at 105–15°, cooled over P_2O_5). The value 0.72 was taken.

3 *Isoelectric Point of Hog Thyroglobulin*—The electrophoretic mobility of hog thyroglobulin was studied in different buffer solutions by the method of Tiselius (3). Acetate buffers (0.02 M NaOAc + x M HOAc), in which x varied from 0.003 to 0.5, and phosphate buffers with constant ionic strength, ($\mu = 0.02$) were used. Before each run the protein solution was made up with the buffer to be used and was dialyzed against the buffer solution for about 15 hours at room temperature. The Swedish hog thyroglobulin was used in all the experiments in a concentration of about 0.3 per cent. Chlorine and bromine light filters were used as in the velocity runs. The temperature was in all cases $20.00 \pm 0.02^\circ\text{C}$.

In Table I and Fig. 1 are given the values found for the mobility in the different buffers. It is apparent from the figure that the points (circles) representing cathodic migration do not lie on the same smooth curve as the points for the anodic-migrating native thyroglobulin. Since earlier work (1) had indicated that thyroglobulin was rapidly denatured in acetate buffers below pH 4.8 experiments were carried out in which thyroglobulin was first exposed to acid acetate buffers of different pH for 1 day. After this the protein solution was dialyzed against distilled water in order to remove most of the acid acetate buffer and finally against the more alkaline phosphate buffer to be used for the electrophoresis experiment. The results are given in Table II and Fig. 1.

It is evident that the new values (crosses) found in this way for the anodic migration correspond much better with the points representing cathodic migration. It is therefore probable that thyroglobulin undergoes an irreversible electrochemical change (denaturation) in

TABLE I
Electrophoretic Mobility of Hog Thyroglobulin

Run No	pH	Buffer	Migration	$\mu \cdot 10^5$
12	3.27	Acetate	Cathodic	14.6
5	3.47	Acetate	Cathodic	13.5
6	3.96	Acetate	Cathodic	10.4
11	4.24	Acetate	Cathodic	8.2
3	4.93	Acetate	Anodic	4.1
17	5.13	Acetate	Anodic	5.5
10	5.30	Acetate	Anodic	7.7
9	5.46	Phosphate	Anodic	9.4
4	5.49	Acetate	Anodic	9.3
1	5.95	Phosphate	Anodic	12.3
13	6.36	Phosphate	Anodic	15.5
18	7.02	Phosphate	Anodic	16.1
2	7.45	Phosphate	Anodic	18.1
19	8.71	Phosphate	Anodic	19.6

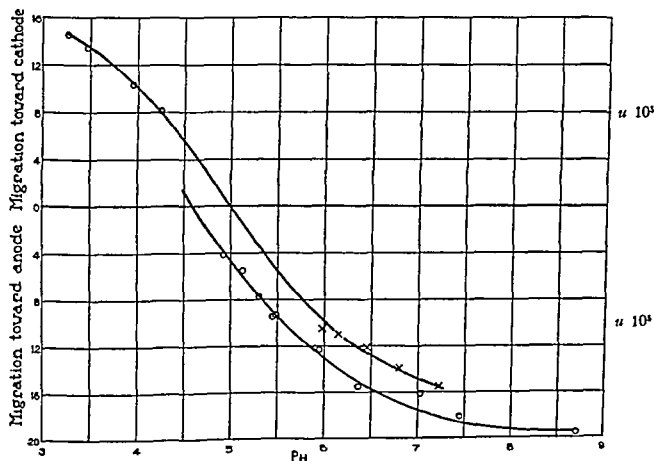


FIG 1 Electrophoresis of thyroglobulin O = points from Table I x = de-natured thyroglobulin (Table II)

the more acid acetate buffers so that the curve formed by the crosses and the original points representing cathodic migration describes the electrophoretic behavior of denatured thyroglobulin. In this connection one of us (4) has found that the isoelectric point is generally shifted toward the alkaline side in the denaturation of proteins.

Denaturation of thyroglobulin also occurs at room temperature in citrate-hydrochloric acid buffers at pH 3.5 (the solution remains clear) 3.9, 4.5 and 4.8 (very slowly) so that the effect is not a specific property of the acetate ion but is probably due to hydrogen ion.

There is also another difference between native and denatured thyroglobulin. The native protein is soluble in acetate buffers down to about pH 4.8 below which denaturation occurs. Denatured thyro-

TABLE II
Denaturation of Hcg Thyroglobulin

Run No.	pH I	pH II	$\alpha \times 10^3$
8	4.4	5.98	10.5
7	4.4	6.15	11.0
16	4.4	6.46	12.1
14	4.2	6.80	13.0
20	5.3*	7.02	16.5
15	5.3	7.25	15.5

* Phosphate.

globulin is practically insoluble in acetate buffers from 4.3 to about 5.5 and in phosphate buffers as far as pH 5.9. The isoelectric point of native thyroglobulin is at pH 4.58 and the slope of the curve at the isoelectric point is $\left(\frac{d\eta}{d\text{pH}}\right)_c = 11 \times 10^{-5}$. For the denatured thyroglobulin the corresponding values are pH = 5.0 and $\left(\frac{d\eta}{d\text{pH}}\right)_c = 11 \times 10^{-5}$. Both the native and the denatured thyroglobulin were electrochemically homogeneous.

4. *Sedimentation Constant of Thyroglobulin*.—By means of the ultracentrifugal method (5) the rate of fall of the molecules can be determined. The sedimentation constant is the velocity in unit field

$$s = \frac{c\pi}{c_0} \frac{1}{\omega^2 r} \quad (1)$$

TABLE III
Sedimentation Velocity of Thyroglobulin

Run No	Thyroglobulin concentration	Solvent			pH of solution	$S_{20} \times 10^{13}$
		μ	μ	μ		

1 Hog thyroglobulin prepared in the United States in 1932						
	<i>pc cent</i>					
12	0 16	NaCl 0 043	NaOAc, 0 057	HOAc 0 041	4 77	20 2
10	0 16		Na ₂ HPO ₄ 0 006	KH ₂ PO ₄ 0 094	5 76	19 3
5	0 16		Na ₂ HPO ₄ 0 05	KH ₂ PO ₄ 0 05	6 75*	18 5
1	0 15	NaCl, 0 1				18 8
8	0 16		Na ₂ HPO ₄ 0 095	KH ₂ PO ₄ 0 005	8 01*	19 3
9	0 16		Na ₂ B ₄ O ₇ 0 05		9 03	18 9
11	0 16		Na ₂ CO ₃ 0 038	Na ₂ B ₄ O ₇ 0 012	9 83	20 2

2 Freshly prepared Swedish hog thyroglobulin						
4	Total protein 0 9 Thyroglobulin 0 7	NaCl, ca 0 1		HOAc 0 133	3 02	15 5
18	0 33	NaCl 0 1		KH ₂ PO ₄ 0 05	6 8†	(10 6)
14	0 17		Na ₂ HPO ₄ 0 05	KH ₂ PO ₄ 0 05	6 8†	19 1
17	0 50		Na ₂ HPO ₄ 0 05	Na ₂ HPO ₄ 0 042	11 33	18 2
15	0 17		NaOH, 0 015		12 02	18 6
20	0 17	NaCl 0 083	NaOH 0 012		12 05	12 4 and 9 2
16	0 17		NaOH 0 012			(6 9)

3 Human thyroglobulin						
2	0 36	NaCl, 0 1		KH ₂ PO ₄ 0 05	6 75*	19 7
6	0 36		Na ₂ HPO ₄ 0 05	KH ₂ PO ₄ 0 005	8 01*	18 4
7	0 36		Na ₂ HPO ₄ 0 095			18 3

* pH of solvent

† Approximate calculated pH

where $\frac{d\lambda}{dt}$ = observed rate of fall, ω = angular velocity, and λ = distance from center of rotation

The data for the runs made are summarized in Table III and Fig 2. Practically all of the runs were carried out at speeds of about 40,000 R P M, corresponding to centrifugal forces of about 100,000 times gravity. Under these conditions, and throughout the pH stability range it was found convenient to take photographs every 10 minutes.

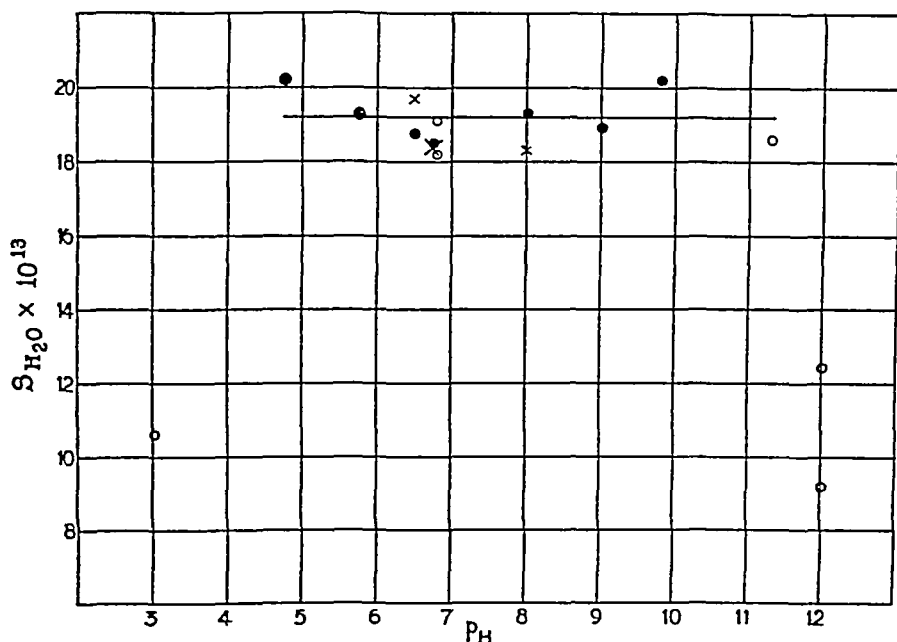


FIG 2 Sedimentation constant of thyroglobulin ● = U S hog thyroglobulin, ○ = Swedish hog thyroglobulin, × = human thyroglobulin. Line indicates mean value of 19.2

Thyroglobulin absorbs ultraviolet light very strongly, so that it was possible to use very dilute solutions. The thickness of the column of solution varied from 1.5 to 6.0 mm, depending on the concentration used. The source of light was a mercury lamp, with chlorine and bromine filters interposed, so that the light used was of wave length below about 270 mμ. In the case of the crude hog thyroid extract, run 4, the opacity of the extract to ultraviolet light rendered the use of the refractometric method worked out by Lamm (6) obligatory.

The experiments were carried out at about 25°C, but the sedimentation constant was corrected for the density and viscosity of the salt solution to a basis of sedimentation in pure water at 20°C according to the formula

$$s_{20^\circ} = s \frac{\eta (1 - V \rho)}{\eta_w (1 - V \rho_w)} \quad (2)$$

where η = viscosity of solvent, η_w = viscosity of water at 20°C, V = partial specific volume of solute, ρ = density of solvent, ρ_w = density of water at 20°C

The sedimentation curves show that the main part (4/5 or even more in the concentrated solution, e.g. run 17, Table III, Fig. 3) consists of particles probably of the same size. However, all the curves indicate the presence of varying amounts of particles with both higher and lower sedimentation constants. The two runs 14 and 17 made at the same pH, but at concentrations of 0.17 and 0.5 per cent, respectively, seem to indicate that most of the lower molecular components present are formed by dissociation of the principal molecular species. This is well illustrated by the differences in the lower portion of the photometer curves for these two runs given in Figs. 3 and 4.

In run 16, at pH 12, insufficient electrolyte was present. Owing to the Donnan effect an apparent single sedimentation constant of $6.9 \cdot 10^{-13}$ was obtained. On the other hand run 20, in which sufficient electrolyte was present, showed that at pH 12 the molecules with the sedimentation constant $19.2 \cdot 10^{-13}$ had disappeared and were replaced by two new molecules with the sedimentation constants 12.4 and $9.2 \cdot 10^{-13}$. Sedimentation constants of nearly these values have many times been found in Upsala especially in pathological sera (von Mutzenbecher (7) and McFarlane (unpublished)). In run 15, at pH 11.3, about 1/3 probably consisted of two lower molecular components, but the curves were difficult to analyze. In run 18, at pH 3, there were at least two molecular species present (probably, too, with sedimentation constants of about 9 and 12.5), but the curves were very difficult to analyze, and therefore only the mean value $10.6 \cdot 10^{-13}$ is given.

5 Molecular Weight of Thyroglobulin from Sedimentation and Diffusion Constants—Svedberg (8) has shown that in cases in which at

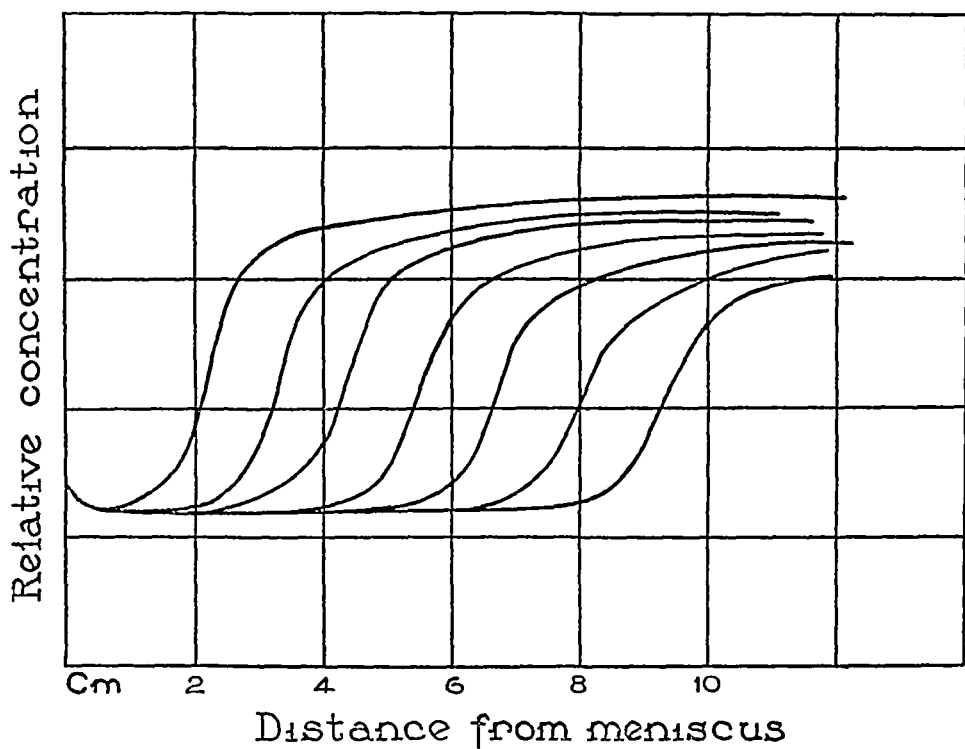


FIG 3 Photometer curve of run 17

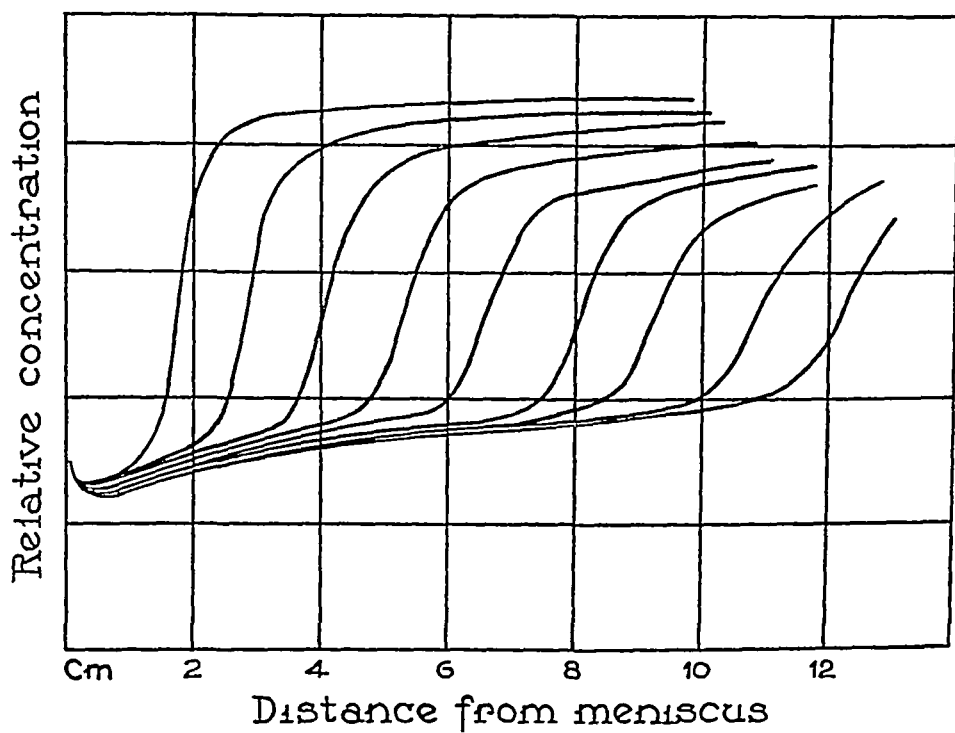


FIG 4 Photometer curve of run 14

could be assumed that the frictional coefficient is the same for a sedimenting particle as for a diffusing particle the molecular weight may be calculated by means of the following formula, independently of the shape of the particle

$$M = \frac{RT s}{D(1 - V\rho)} \quad (3)$$

in which s is the sedimentation constant and D the diffusion constant at the temperature T (abs), R the gas constant, V the specific volume, and ρ the density of the solution

Mr A G Polson, who is carrying out a systematic series of diffusion determinations on different proteins, has made some preliminary diffusion experiments with hog thyroglobulin and kindly put his values at our disposal. These experiments were performed in the apparatus used by Tiselius and Gross (9) but the measurements were made by means of the refractometric method (6) which Lamm has recently adapted for diffusion experiments

The diffusion measurements were made with a concentration of thyroglobulin of 1 per cent and at a pH of about 5.5. The temperature was 20°C and the values obtained in the salt solutions were corrected to water at 20°C. The diffusion curves were not quite ideal, indicating also that thyroglobulin is not monodisperse, if the diffusion constant is calculated as for a monodisperse substance, a value of $2.0 \cdot 10^{-7}$ cm²/sec is found. Mr Polson has, however, analyzed the curves from point to point and found that most of the material has a diffusion constant of $2.39 \cdot 10^{-7}$, but that particles are present with both lower and higher diffusion constants. By formula (3) the latter value and $19.2 \cdot 10^{-13}$ for the sedimentation constant lead to a molecular weight of about 700,000 (actually 696,000).

6 Sedimentation Equilibrium Runs—The ultracentrifugal technique allows of a direct determination of the molecular weight by means of sedimentation equilibrium measurements (5). Six equilibrium runs were made with different concentrations of hog thyroglobulin and different buffers. The speed varied between 2,500 and 3,300 R P M. Equilibrium was attained after 3 to 5 days.

The measurements of the concentration gradient were made by means of the refractometric method (Lamm (6)), but calculation of

the molecular weight from the measurements was carried out by a new and as yet unpublished method worked out by the junior writer in which the following expression is used

$$M = \frac{RT \frac{dc}{dx}}{(1 - V \rho) \omega^2 x c} \quad (4)$$

in which ω is the angular velocity of the centrifuge and $\frac{dc}{dx}$ and c are the concentration gradient and the concentration at the distance x from the center of rotation. Now according to the refractometric method (4) the measured displacement of the lines, Z , is determined by

$$Z = k \frac{dn}{dx} = k \alpha \frac{dc}{dx} \quad (5)$$

in which k is an apparatus constant depending on the thickness of the cell, the distance from the scale to the cell and the photographic enlargement used, $\frac{dn}{dx}$ is the refractive index gradient, and α is the refractive index increment. By introducing this expression containing Z in place of $\frac{dc}{dx}$ in formula (4) we get

$$M = \frac{RT Z}{(1 - V \rho) \omega^2 x c k \alpha} \quad (6)$$

In order to evaluate c it is assumed that the total amount of protein (substance) in solution at the start is still present in solution when the equilibrium is measured. It is then possible to determine the concentration at different points in the cell, for instance, by a modification of the method used by one of us (10)³. If some of the substance present at the start is precipitated this method will give somewhat low values. Details of the method will be published elsewhere.

In these runs measurements (photographs) were taken with the Hg line 436 m μ and with a "Lifa" filter No 216 (630–685 m μ), except in the first run. The refractive index increments were in part deter-

³ Pedersen, K. O., *Z. phys. Chem., Abt. A*, 1934, **170**, 52

mined by Mr Kjell Andersson For $\lambda = 436 \text{ m}\mu$, $\alpha = 192.8 \cdot 10^{-5}$ and for $\lambda = 656 \text{ m}\mu$, $\alpha = 186 \cdot 10^{-5}$ The calculated values for violet and red light and for different scale distances agreed fairly well

Phosphate buffers were used in a concentration of 0.1 to 0.15 molar total phosphate, so that the Donnan effect may be neglected

Below is given a summary of the results The concentrations are given in grams per 100 ml solution

RUN I

c at Start = 0.335

Rev Per Sec 55.0

pH = 6.8

x	c	M
5.60	0.160	554,000
5.65	0.197	565,000
5.70	0.245	587,000
5.75	0.307	606,000
5.80	0.388	619,000
5.85	0.493	623,000

RUN II

c at Start = 0.335

Rev Per Sec 53.9

pH = 6.1

x	c	M
5.60	0.164	470,000
5.65	0.195	495,000
5.70	0.234	521,000
5.75	0.284	556,000
5.80	0.352	598,000
5.85	0.443	647,000

RUN V

c at Start = 0.970

Rev Per Sec 42.5

pH = 6.6

x	c	M
5.55	0.520	667,000
5.60	0.599	656,000
5.65	0.690	649,000
5.70	0.799	653,000
5.75	0.926	684,000
5.80	1.092	(790,000)
		662,000

RUN VI

c at Start = 0.970

Rev Per Sec 49.5

pH = 6.6

x	c	M
5.55	0.421	686,000
5.60	0.513	657,000
5.65	0.623	638,000
5.70	0.755	627,000
5.75	0.915	630,000
5.80	1.115	661,000
5.85	1.390	(772,000)
		650,000

Runs III and IV are omitted since they yielded the most uncertain values In the other cases the error is probably about 10 per cent, and is largely due to the particles of higher molecular weight present,

which made the calculation of the concentration at the bottom of the cell difficult

From these equilibrium runs it is obvious that the thyroglobulin has a very strong dissociation tendency in more dilute solutions (below 0.5 per cent). This agrees very well with the results of the sedimentation velocity runs. In the more concentrated solutions the dissociation of the thyroglobulin is not very marked, but just as in the dilute solution there are some particles of higher molecular weight present.

7 Molecular Frictional Constant—The molecular frictional constant may be calculated according to Svedberg (11) from the following formula

$$f = \frac{M(1 - V\rho)}{s} \quad (7)$$

If 675,000 be taken as the best value for the molecular weight of thyroglobulin, this leads to $9.9 \cdot 10^{16}$ for the molecular frictional constant. For a spherical particle the molecular frictional coefficient is determined by the formula

$$f_0 = 6\pi\eta N \left(\frac{3MV}{4\pi N} \right) \quad (8)$$

in which N is the Avogadro number and η is the viscosity of the solution. For $M = 675,000$, $f_0 = 6.58 \cdot 10^{16}$. Since the dissymmetry number is defined as $\frac{f}{f_0}$ its value for thyroglobulin is 1.50.

DISCUSSION

The principal component of thyroglobulin is characterized by a high sedimentation constant, $s_{20} = 19.2 \cdot 10^{-13}$. Of the proteins of the higher animals already studied only a small serum globulin fraction with approximately the same s has been reported (7). From run 4 it would appear that thyroglobulin is actually present in the gland in such large molecules, for the original crude extract showed a sedimentation constant consistent with the findings on the purified protein. The somewhat lower value of s found was doubtless in large measure due to the high concentration of protein in the crude extract (*cf.* Reference 4). If, as appears probable, thyroglobulin is the actual thyroid hormone,

it is a hormone of molecular weight 675,000. This is twenty times as large as that found for insulin (12), so that there is a large gap between the two protein hormones of which the molecular weights are known. It is perhaps too early to judge the physiological significance of the high molecular weight of thyroglobulin except insofar as it would render difficult of acceptance any theory of thyroid hormone action based on the direct diffusion or penetration of the hormone into the cell, assuming the protein to be the actual hormone. It is, of course, possible that a large protein molecule such as thyroglobulin could be deposited on the surface of a cell, there either to exert its action directly by means of the two or three tyroxine groupings present per molecule, or to be broken down into reactive fragments by the proteolytic enzymes of the cell. Possibly these processes would be furthered by the tendency of thyroglobulin to dissociate in dilute solution.

Chemically there is much of interest in the high molecular weight of thyroglobulin. Outside the domain of the respiratory proteins it is perhaps the most easily accessible protein of its size and is therefore readily available for studies on the problems arising in connection with large molecules not containing metallic groups. Hog thyroglobulin is also characterized by a far lower isoelectric point, at pH 4.58, than those of the known serum globulins. While this may be a peculiarity of the animal from which the protein was derived it is evident that in the comparatively alkaline body fluids thyroglobulin would be highly ionized. Thyroglobulin exhibits a pH stability range similar to that of other proteins, but its dissociation tendency on dilution seems to be much greater. It also resembles the serum proteins in that its molecule is not spherical. The low specific volume, however, is unusual.

Although thyroglobulin which has once been dried is difficult to redissolve, the purified protein is remarkably stable in solution. Thus a preparation kept in solution for 2 years showed the same sedimentation constant for its principal component as did a freshly prepared portion. This component was, however, present in smaller amount. It is also noteworthy that the chief component of human thyroglobulin showed essentially the same sedimentation constant as did hog thyroglobulin, so that the molecular weights of the two proteins may be considered as not greatly different.

SUMMARY

- 1 The sedimentation constant of hog thyroglobulin is $19.2 \cdot 10^{-13}$. That of human thyroglobulin is essentially the same.
- 2 The specific volume of hog thyroglobulin is 0.72.
- 3 The isoelectric point of native hog thyroglobulin is at pH 4.58, that of denatured thyroglobulin at pH 5.0.
- 4 The molecular weight of hog thyroglobulin is, in round numbers, 700,000, as calculated from the sedimentation and diffusion constants, or 650,000, as calculated from the sedimentation equilibrium data.
- 5 The thyroglobulin molecule deviates markedly from the spherical.

In conclusion the senior writer wishes again to express his hearty thanks to Professor The Svedberg for his generous extension of the hospitality of his laboratory and his freely given counsel, and to the other members of Professor Svedberg's staff for their assistance and many courtesies.

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THE ELECTRICAL ACTIVATION OF PASSIVE IRON WIRES IN NITRIC ACID

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The chief analogies between the activation of a passive iron wire by the electric current and the electrical stimulation of an irritable living tissue may be stated briefly as follows ¹

In both cases the activating or stimulating current must exceed a certain minimum as regards (1) intensity (referred to unit surface, or density), (2) duration of flow, and (3) rate of change. The polar rule also applies, to be activated the passive wire must be the cathode, if it is the anode, the effect of the current is stabilizing, *i e* is in the direction of passivation rather than activation, thus a passive wire (*e g* in 70 volumes per cent HNO_3)² polarized anodically by a constant current is more difficult to activate (*e g* mechanically or electrically) than an unpolarized wire under the same conditions, and transmits an activation wave more slowly ¹. Conversely, a wire polarized cathodically, by a current of "sub threshold" density, is more readily activated and transmits activation more rapidly than an unpolarized wire ³. Interferences and facilitations of this kind are to be regarded in the physical sense as summation effects, they have thus physiological analogies in the phenomena of electrotonus ⁴. Summation of sub-threshold "stimuli," mechanical and electrical is also shown ¹. Finally, the passive wire may be activated, under appropriate conditions, by the break of an already flowing constant current (see below, p 122)

¹ For a fuller general comparison *cf* Lillie R. S., *J Gen Physiol*, 1920, 3, 107, 129

² Volumes of HNO_3 of specific gravity 1.42 in 100 volumes of solution

³ For the effects of polarization on nerve *cf* Bishop, G. H. and Erlanger J., *Am J Physiol*, 1926, 78, 630

⁴ *Cf* Ebbecke U. Zur Lehre vom Elektrotonus *Ergebn Physiol*, 1933, 35, 756

Since Hermann's time the primary change in electrical stimulation has been very generally recognized to be a change of polarization. This change determines or releases the characteristic biological reaction of stimulation. Apparently, however, it is not sufficient that a certain critical change of polarization should be produced by the stimulating current, the polarized state must be reached with a certain minimal rapidity and maintained for a certain minimal time. As is well known, the minimal duration of current flow required for stimulation by a constant current of threshold intensity varies characteristically for different tissues and for the same tissue under different conditions. With stronger currents the required duration decreases as intensity increases, and various formulae have been proposed to describe the intensity-duration relationship.⁵ If polarization is the primary change in electrical stimulation, the total duration necessary for any stimulating current may be regarded as the sum of two separate durations, (1) that of the purely physical process of polarization and (2) that of the succeeding presumably electrochemical process which initiates the biological reaction. These may be characterized respectively as polarization time and activation time. In certain oscillograph records of electrical stimulation the special characteristics of the two corresponding processes can be clearly distinguished, a curve having the general form of a polarization curve is followed immediately by another showing peculiarities characteristic of the biological response.⁶

Recently the phenomena of polarization have been studied by Ebbecke⁷ in a simple physical model consisting of a pair of platinum wires immersed in an electrolyte solution and connected through a galvanometer to a rheostat and battery. With potentials lower than the decomposition potential of the solution, current flows temporarily on making and breaking the current, but not (or inappreciably) during the intervening period. Similarly, little or no current flows if the potential (within the defined range) is slowly changed. In these several respects a wire immersed in a solution acts like a condenser, and in further studies with condenser systems Ebbecke⁸ has shown that the characteristic duration-intensity curves of electrical stimulation agree well with the conception of stimulation as being determined by the charging or polarizing of surfaces with properties similar to those of imperfectly insulated condensers. This general view has been reached independently by many other investigators.⁹

⁵ For a recent full account cf. Monnier, A. M., *L'excitation électrique des tissus*, Paris, Hermann et Cie, 1934.

⁶ Cf. Bishop, G. H., *Am J Physiol*, 1928, 84, 417, Fig. 6, Schmitz, W., and Schaefer, H., 1933, *Arch ges Physiol*, 233, 229, Fig. 2.

⁷ Ebbecke, U., *Arch ges Physiol*, 1926, 211, 485.

⁸ Ebbecke, U., *Arch ges Physiol*, 1927, 216, 448.

⁹ Cf. Lapicque, L., *L'excitabilité en fonction du temps*, Paris, Presses universitaires de France, 1926, Monnier, A. M., see footnote 5, Bishop, G. H., *Am J Physiol*, 1928, 85, 417, Ebbecke, U., see footnote 4, Hill, A. V., *Chemical wave transmission in nerve*, Cambridge University Press, 1932.

If in Ebbecke's model we replace the platinum wires by passive iron wires (with HNO_3 as electrolyte) the physical conditions remain essentially unchanged until a certain upper limit of potential is reached, then the cathodal wire is activated, and the characteristic electromotor variation and other features of activation are shown. As already described,¹ the reaction of the wire varies in its special features and time relations according to a number of conditions, including the kind of wire, concentration of acid, temperature, and presence of other substances. In more concentrated HNO_3 (above 55 v per cent) it resembles the response of irritable living tissues in being temporary and automatically reversed, the wire shows a darkening and effervescence lasting for a brief period (varying from a fraction of a second to several seconds) followed by a return to the passive state. In acid of this concentration the passive state is a stable one (analogous to a physiological 'resting' state) while the active state is unstable or temporary and maintainable only intermittently—hence the tendency to automatic rhythm shown under certain conditions.¹⁰ This characteristic behavior is based on the fact that two chemically opposed processes are concerned in the reaction cycle of activation. The first is the reduction which breaks down the passivating film, this effect is associated with automatic transmission by local circuits (activation phase) the second is the oxidation which reforms the film (repassivation or recovery phase). After repassivation secondary changes in the film proceed for some time longer, occupying the so called relative refractory period.¹¹

Certain characteristic features of electrical activation depend on the relative rates or intensities of these two opposed processes. A current of a certain intensity and duration will activate a wire in nitric acid of a certain concentration (e.g. 60 v per cent) but not in more concentrated (e.g. 70 v per cent) acid. Since the same quantity of current traverses the surface of the wire in both cases, it is evident that some factor opposing activation is present which is effective in the stronger but not in the weaker acid. This factor is the oxidizing action of the HNO_3 which increases with concentration. By increasing the current the wire can be activated in the stronger acid. Reduction then becomes temporarily more rapid than oxidation and the film is disrupted sufficiently to activate the whole wire. The all-or none characteristic, so conspicuous in fully recovered wires, depends on the automatic transmission by local circuits. Such transmission always plays a part in any case of activation, which may be initiated in a small local area and yet involve the whole wire.

Such experiments show that the iron wire in nitric acid represents a system where activation, an effect of reduction, is opposed by a counter process, oxidation whose effect increases with the external concentration of acid, this counter process acts by forming an insoluble oxide which is deposited as an impermeable surface film. In strong acid the process of repassivation is automatically initiated

¹⁰ Lillie R. S., *J Gen Physiol*, 1929, 13, 1

¹¹ Lillie R. S., *J Gen Physiol* 1931 14, 349

at any region as soon as that region becomes active and therefore anodal¹² The system possesses in fact the general properties ascribed by Blair¹³ to his recent mathematico-physical model of excitation, in which the excitatory process is automatically opposed by a counter-process proportional to the state of excitation

Another peculiarity shown by both the passive wire and the living tissue is that the readiness with which either system responds to any activating agent varies in a characteristic manner during a certain interval of time ("refractory period") immediately following a response Responsiveness is slight or absent at first and then increases progressively to a maximum corresponding to the normal equilibrium or resting state of the system In both systems "absolute" and "relative" refractory periods are present^{1,11} and the temperature coefficients of recovery are similar¹⁴ During its relative phase the wire requires for activation a stronger or more prolonged current than when fully recovered, and the transmission of activation is slower¹⁴ It is therefore necessary, in studying experimentally the intensity-duration relationship in passive wires, to make all observations either in fully recovered wires, or in partially recovered wires at equal intervals between successive activations The wires used must be uniform in composition, since the rate of recovery varies greatly in different kinds of iron, and temperature must be constant

Since activation depends on the reduction of the oxide film, it is to be assumed that a certain minimal PD between the cathodal wire (or the initiatory local region in such a wire) and the adjacent layer of solution must be reached in order to activate This is the decomposition potential characteristic of the system, electrons then pass from the metal to the reducible oxide molecules at its surface and reduction occurs This potential is not reached instantaneously when the circuit is closed but rises to the required level progressively, following a curve similar to the charging curve of a condenser After the establishment of this critical potential, current flows continuously between the metal and the electrolyte, and the equivalents reduced are proportional to the Faradays passed It seems clear that in the living system also a chemical reaction is initiated at the surface of the irritable element after the polarizing current has flowed for a certain time, and that this reaction directly or indirectly alters the plasma membrane, apparently increasing its permeability To regard this reaction as an electrolysis might be unjustifiable, unless the protoplasmic surface is equivalent to an electrode surface It is, however, well known that a thin electrically polarized membrane separating two solutions may be the seat of chemical reaction when a current

¹² It should be remembered that in the iron wire this condition holds only in strong acid (>55 v per cent), in weak acid the passive state is one of unstable equilibrium, and activity once initiated continues until the metal is completely dissolved

¹³ Blair, H A, *J Gen Physiol*, 1932, 15, 709, 731

¹⁴ Lillie, R S, *J Gen Physiol*, 1925, 7, 473 Cf p 500

is passed, this is the phenomenon of electrostenolysis, which has been recently reinvestigated with an improved apparatus by E S Fletcher¹⁵ in this University. He has shown that while oxidation and reduction occur when a current is passed through a thin membrane (e g, of cellulose acetate) interposed between solutions containing oxidizable or reducible substances (provided there is a sufficient fall of potential between the two faces), there is this characteristic difference between the membrane and a metallic electrode, that in electrostenolysis (e g, in the oxidation of Fe^{++} to Fe^{+++}) the ratio of Faradays passed to equivalents oxidized is not unity but is typically of the order of some hundred to one. Allowing for this difference we may consider the conditions at a membrane and at an electrode as comparable, the two are alike in the respect that in both cases the chemical effect depends on the passage of current across a surface polarized to a sufficient potential. This similarity leads us to expect that the general quantitative conditions of electrical activation will be similar in the living tissue—where the primary effect of stimulation appears to be a chemical reaction at a membrane—and in the iron wire model. The electrostenolysis model is no doubt far simpler than the actual conditions in living tissues, but the same is evidently true of all physical (or conceptual) models of vital processes.

Intensity Duration Relation in Passive Iron

When a constant current is passed between two passive iron wires immersed in a bath of nitric acid the chief general factors determining whether or not activation will occur are (1) E M F of current, this must usually be of the order of 1 volt or more for a regular effect, (2) density of current (i_e , amperes per unit area of wire), (3) duration of flow, (4) concentration of acid, (5) temperature, and (6) interval since the previous activation of the same wire (i_c , duration of recovery period). There are also factors depending on the special properties of the iron used, accidental peculiarities in the mosaic structure of the surface are a source of variation, since activation of the whole wire may result from a small local effect which is propagated, thus if any region has a greater than average susceptibility to reduction, the readiness with which the whole wire responds to an electric current is increased. Such local nuclei may be so small that in wires kept in dilute (e g, 10 v per cent) HNO_3 a kinetic or statistical factor seems to enter, shown in a tendency to irregular spontaneous activation.¹¹ In single experiments such accidental factors may be of importance in determining the critical or "threshold" level (of

¹⁵ Fletcher E S Ph D dissertation, A quantitative study of electrostenolysis University of Chicago, 1934 shortly to be published

intensity or duration) at which activation occurs, it is probably largely for this reason that reproducibility of behavior is found to be less perfect in iron wires than in living nerves or muscles under well controlled conditions

Experiments have been carried out as follows

In these experiments pure iron wire (the so called Armco brand) *ca* 2 mm in diameter was used.¹⁶ Two lengths of wire (anode and cathode in the experiment) are bent to an appropriate shape and attached to movable keys (*e g*, the Harvard cross-circuit type) supported on stands on either side of the vessel containing the acid, usually this was a flat-bottomed "finger bowl," 10 cm in diameter and containing 300 cc. of acid. The wires were coated with paraffin¹⁷ except at their free ends which were straight and bare for equal lengths (usually 5 or 6 cm), these were immersed (after passivation in strong HNO_3) horizontal and parallel to each other in the acid about 4 cm apart. The area of metal exposed to the acid¹⁸ and the distance between the wires were thus constant during a series of experiments. Such an exposed length of wire remains permanently passive in strong nitric acid if undisturbed, on activation it shows a single brief reaction, with darkening and effervescence lasting, in 70% per cent HNO_3 at 20°, for about 1 second.

The keys supporting the wires were connected through a tube rheostat (for varying the potential) and pole-changer to a storage battery of four to six cells with a total *E.M.F.* of 6-9 volts. In each series of experiments currents of known *E.M.F.* (led to the wires from the rheostat) and known durations were passed through the circuit containing the passive wires. At any given *E.M.F.* it is found that the current must flow for more than a certain minimal length of time in order to activate the cathodal wire. For obtaining brief durations of current (from 0.5 to 30 σ) a Lucas pendulum (vibrating spring interruptor) was used, this was calibrated by a ballistic galvanometer in the Physical Laboratory. It was found that for the greater part of its excursion the curve of vibration of such a spring corresponds closely to a sine curve. In a number of experiments the vibration rate of the spring was decreased by attaching weights, the time range could

¹⁶ The wire used in my previous study on rhythmical reactions (*J Gen Physiol*, 1929, 13, 1). It contains 99.8 per cent iron and less than 0.2 per cent carbon, according to analyses furnished by the manufacturers.

¹⁷ A wire unprotected by paraffin shows irregular action where it passes from the solution into the air.

¹⁸ A thin layer of wire is dissolved away at each activation and allowance must be made for this. At intervals during a series it is often necessary to repair the paraffin coating at its boundary with the bare wire, otherwise acid enters between paraffin and metal and sets up irregular or rhythmical action.

thus be lengthened to ca 90 σ . For still longer durations of current, up to 1.3 seconds the spring of the Lucas apparatus was removed and the two keys (respectively opening the cross circuit between the passive wires and breaking the battery circuit) were actuated by a rigid arm attached to a uniformly rotating axis with a period of 20 seconds operated by an electric motor and gearing.

In any typical series of experiments the least duration of current was determined at which each wire, with the rheostat placed at a known potential showed regular activation. Conditions of temperature, concentration of acid, length and thickness of the exposed wire, and duration of recovery period (interval since the previous activation of the same wire) were kept uniform. In most cases the current was passed between the wires at regular intervals of 30 seconds, the pole-changer being reversed after each current; the interval between successive stimulations' of the same wire was then exactly 1 minute. In 70 v per cent HNO_3 (at 20) recovery is almost complete after this interval. The threshold condition for each wire, with each potential used is given by the least duration at which the wire responds to the current every time in several successive trials. If the duration is decreased still further (e g from 7 σ to 5.7 σ in some of the experiments summarized in Table I), it frequently happens that the wire responds regularly at every alternate passage of the current, i e with a recovery period of 2 minutes but not of 1 minute, indicating a slight increase in responsiveness during the second minute of recovery. Conversely, if the recovery period is shortened e g to $\frac{1}{2}$ or $\frac{1}{3}$ minute, the required duration of the activating current is correspondingly increased. The course of the recovery curve can thus be followed (cf Table III).

A typical example of a series of determinations carried out in this manner is given in Table I.

The third column of Table I gives for both wires the product of the intensity (taken as proportional to voltage) into the least effective duration of current (it), the fourth column gives the product of the intensity into the square root of the duration ($i\sqrt{t}$), in accordance with Nernst's well known formula. It will be noted that the Nernst product shows for the first five or six determinations a fair approach to constancy, afterwards it falls off, and with the weaker and more prolonged currents (omitting Experiment 9) the direct product, it , is more nearly constant. This behavior has been found typical in a large number of experiments. When the current is still further decreased activation tends to be delayed so that both products increase again, with further decrease the response becomes irregular or fails. Values corresponding to the physiological concepts of rheobase and chronaxie can be estimated from the course of the intensity-duration

TABLE I

Six storage cells, temperature 21°, 70 v per cent HNO₃ Length of exposed portions of wire 6 cm Recovery interval 1 minute

E M F of activating current (per cent of total rheostat potential)	Least effective duration of current (σ)		it		$i\sqrt{t}$	
	Wire A	Wire B	Wire A	Wire B	Wire A	Wire B
(1) 90	7 0	7 5	630	685	238 5	243
(2) 80	9 2	9 5	736	760	244 0	248
(3) 70	11 3	12 0	791	840	235	242
(4) 60	12 7	15 1	762	906	213	234
(5) 50	16 6	19 0	830	950	205	218
(6) 40	22 8	27 0	912	1080	190	208
(7) 30	30 1	33 3	903	1002	165	173
(8) 25	40 8	53 4	1020	1445	160	183
(9) 20	ca 92 0	ca 92 0	1840	1840	192	192

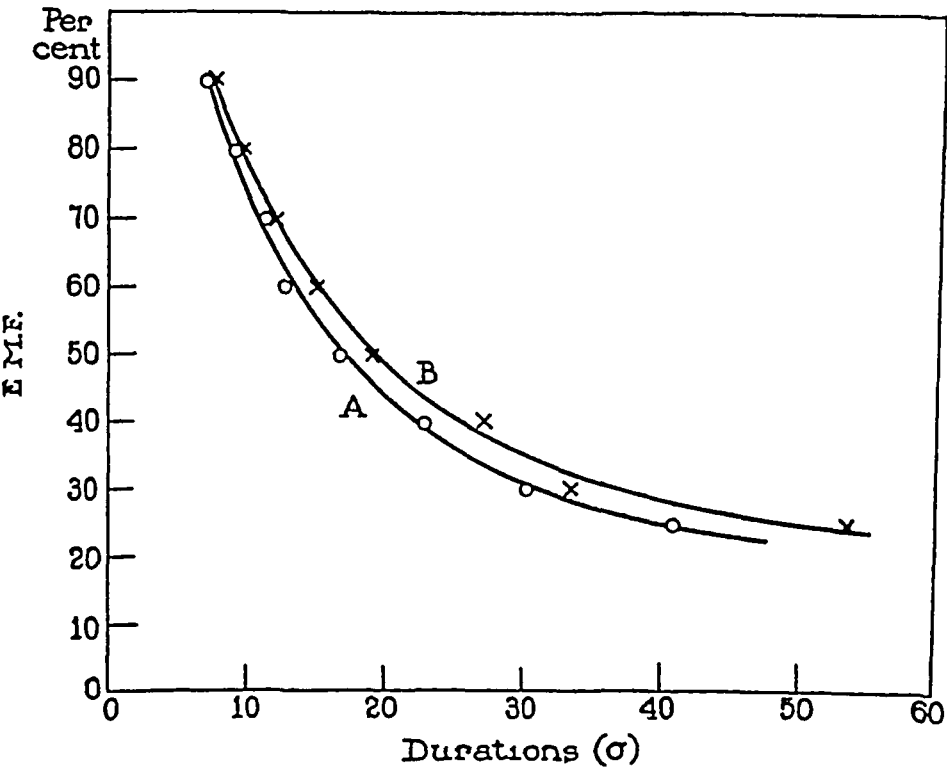


FIG 1 Intensity-duration curves for wires A and B of Table I Ordinates, voltages in percentage units, abscissae, durations of current in σ

curve (Fig. 1), their precise magnitudes vary with the conditions of the experiment (concentration of acid, area of wire, resistance of circuit, etc). In the above series the rheobase for wire B would be approximately 1.5 volts and the chronaxie 30σ , the former value is clearly an arbitrary one, determined by the steepness of the potential fall between the wires under the special conditions of the circuit.¹⁹ The potential between active and passive wires in 70 v. per cent acid, as measured with a voltmeter, is ca. 0.7 volt, this is greater than the decomposition potential of passive iron, as is shown by the automatic transmission of activation waves.

Extrapolation of the intensity duration curves in Fig. 1 indicates a rheobase of between 15 and 20 (in the percentage units of Table I) for the two wires, wire A being consistently somewhat more responsive than wire B. Since, as already pointed out, the two opposing factors assumed in Blair's¹² discussion of electrical stimulation are actually present in the iron wire system, it is of interest to plot the data in accordance with his formula²⁰

$$\log \frac{V}{V - R} = \Lambda t + C$$

(where V is applied voltage, R voltage of rheobase, t duration of current, and Λ and C constants). On semilogarithmic paper the data fall with fair accuracy along a straight line when the rheobase is taken as either 17.5 or 15. Wire A shows a somewhat better fit with $R = 15$, wire B with $R = 17.5$ (Fig. 2).

The precise value of the constants under the conditions of these experiments has no special significance, but it is a matter of general interest that the form of the intensity duration curve is so closely similar to that found in irritable tissues. In a large number of similar

¹⁹ The i.p. between the wires, with constantly flowing current would be proportional to the ratio of the resistance of this part of the circuit to the total resistance.

²⁰ Hill, A. V.,⁹ p. 59, reaches a similar formula, $\log (1 - \frac{R}{C}) = \Lambda t$, from consideration of a condenser type of model, and shows that it conforms accurately to recent data of Rushton on frog's nerve (Rushton, W. A. II, *J. Physiol.*, 1932, 76, 445).

experiments the Nernst formula has been found to give a good agreement with observation for currents of brief duration (from 2σ to 12 or 15σ), but with longer durations the agreement is less satisfactory (*cf* Tables I and II) Ebbecke⁸ has shown that the process of simple polarization by a constant current, as illustrated by the charging of a

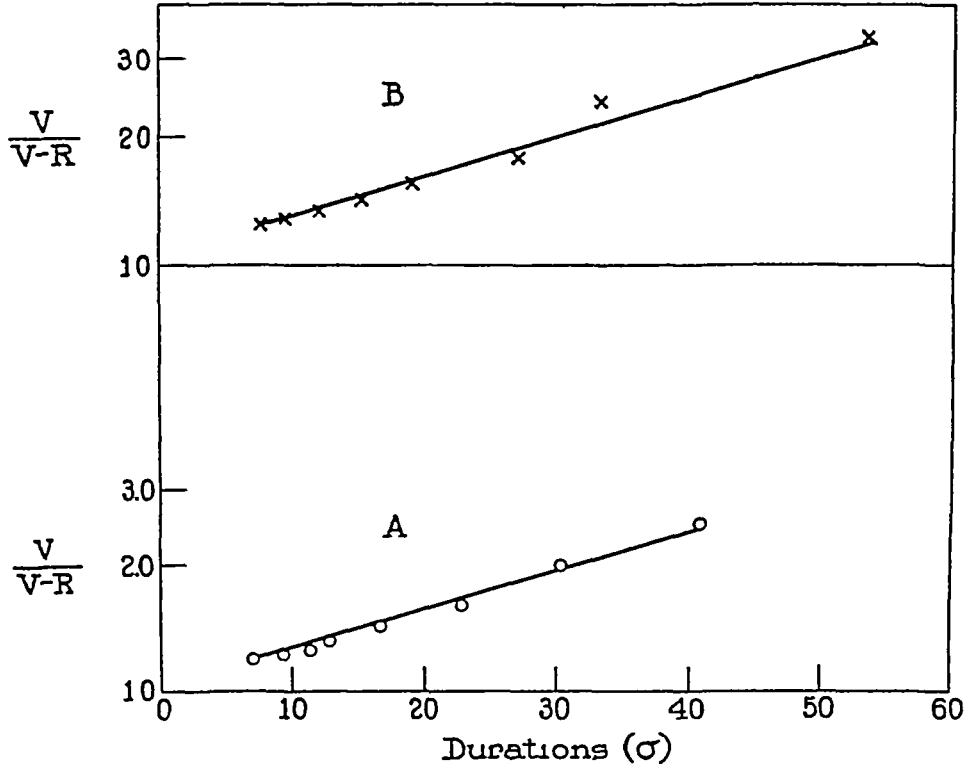


FIG 2 Semilogarithmic diagrams showing linear relation of $\log \frac{V}{V-R}$ to durations V , voltage in percentage units of Table I, R , rheobase, taken as 15 for wire A, as 17.5 for wire B, t , durations of current in σ

condenser, conforms approximately to Nernst's square root formula within a considerable range of potentials, but that above and below this range it has no application. In the case of the iron wire an agreement with the Nernst formula might be expected with the briefer and stronger currents, in which it seems likely that a relatively large fraction of the total duration of current flow is occupied by the process of polarization. After polarization has reached the critical

level required, activation would occur rapidly or instantaneously with strong currents, while with weaker currents the flow of current would continue for a relatively longer time before an activating degree of reduction is attained. During this second period, following the preliminary polarization, the degree of reduction may be regarded as proportional to the product it , as required by Faraday's law of electrolysis. We may thus account for the falling off of the Nernst product $i\sqrt{t}$ with the longer and weaker currents and also for the approach to constancy in the product it during a certain limited range of longer durations. As already pointed out, if the current is still further weakened, the opposing oxidative action of the HNO_3 prevents the

TABLE II
Conditions Similar to Those of Table I Three Storage Cells

E.M.F. of activating current	Least effective duration (ϵ) of current in HNO_3 of			\sqrt{t}		
	80 v per cent	70 v per cent	60 v per cent	80 v per cent	70 v per cent	60 v per cent
<i>per cent</i>						
87.5	6.3	3.1	1.9-2.3	219	154	122-134
75	8.4	4.3-4.6	2.7-3.1	217.5	155-160	124-132
62.5	9.6	6.7-7.2	4.7-5.5	194	162-167	135-141
50	12.6	9.3-9.7	7.2-7.6	175	152-156	134-138
37.5	17.6	13.9-14.4	ca 9.6	158	140-142.5	ca 116
25	(No activa tion)	1 second or more	18.5-19.5			ca 105

disruption of the film from ever reaching a stage sufficient to start an activation wave. The region of sub threshold intensities is then reached, the upper limit of which is determined by the conditions (concentration of acid, etc.) already defined.

Influence of Concentration of HNO_3

For brief durations of activating current the product $i\sqrt{t}$ may be taken as a measure of the resistance of the system to activation. This product shows a characteristic increase with increase in the concentration of acid, as shown by Table II. In these experiments wires were activated, under otherwise uniform conditions, in HNO_3 of the three concentrations, 60, 70, and 80 volumes per cent.

For durations of less than 10σ the average values of $i\sqrt{t}$ in the three concentrations of HNO_3 are approximately for 80 v per cent, 210, for 70 v per cent, 160, for 60 v per cent 130. Since the conductivities increase with concentration of acid, the differences in responsiveness are somewhat greater than these figures represent. The interpretation of these results has already been indicated, increasing the concentration of HNO_3 increases the intensity of the oxidative or passivating action which opposes activation.

Influence of Duration of Recovery

In the experiments of Tables I and II the interval between the successive activations of each wire (the period of "recovery") was uniformly 1 minute. The relation between the duration of the re-

TABLE III

Exposed lengths of wire 6 cm 70 v per cent HNO_3 Temperature 21° Voltage of activating current constant throughout (four storage cells, 5.7 volts)

Interval since previous activation	Minimal duration of activating current (σ)
120	12.7
60	13.8
30	25.2
20	39.0

covery period and the readiness of activation by a current of constant intensity is shown in the experiments of Table III. In these experiments the intervals between the successive activations of each wire were varied between 20 seconds and 2 minutes.

With 20 seconds of recovery the critical duration for activation is three times as long as with 2 minutes. After this interval recovery may be regarded as complete. Since the time occupied by the polarization process is presumably constant in all experiments, we may infer that the critical degree of reduction required for activation is several times greater in a wire exposed to the current immediately after activation than in the same wire after complete recovery. The most probable assumption is that the oxide film is relatively thick immediately after repassivation and later becomes thinned to a constant thickness.

(possibly monomolecular) by the solvent action of the acid¹¹ In this progressive change of the system from a resistant state to a state of maximum alterability (corresponding to an equilibrium or "resting" state) there is an evident parallel with the change in an irritable tissue during the relative refractory period In both cases the change in responsiveness is to be ascribed to a change in the properties of the surface layer In the living system some structural or chemical change in the plasma membrane depending on metabolism is apparently involved, but its precise nature is unknown

Influence of Surface-Active Substances

The presence of surface active compounds also decreases the responsiveness of the iron wire to the electric current and correspond-

TABLE IV

Conditions as before HNO_3 70 v per cent Temperature 21° EMF 6 volts

Concentration of amyl alcohol	Minimal duration of activating current (σ)
<i>volts per cent</i>	
0 (control)	7.2
0.25	8-9.6
0.5	10.5-12.1
0.75	13.8-16
1.0	21.2
1.5	24.0-25.7
2	ca 24.0

ingly decreases the rate of transmission, the latter effect is especially marked during the early part of the relative refractory period For example, in steel wires immersed in 70 v per cent HNO_3 containing 0.5 v per cent amyl alcohol the speed of transmission, after 3 minutes recovery, was found to be only 30 per cent of that shown by the control wire in pure 70 v per cent HNO_3 Such compounds also delay and may even entirely prevent the automatic repassivation Changes in surface conditions, dependent on adsorption of the compound, undoubtedly underlie all such effects In general, the effectiveness of such compounds increases with their surface activity So far, however, no conditions have been found under which a surface

active compound entirely prevents (although it may greatly delay) the transmission of an activation wave. In this respect the parallel with narcosis is incomplete.

Table IV illustrates the manner in which the minimal duration of an activating current is prolonged by the addition of amyl alcohol.

Since the amyl alcohol is rapidly oxidized under these conditions the delay in activation is to be attributed chiefly to the caproic acid which is formed. Butyric acid added to nitric acid acts similarly, although less effectively in equivalent concentrations. These effects are completely reversible on returning the wire to pure nitric acid. Acetic acid has little or no effect, even in much higher concentrations.

Activation by Break of Constant Current

When a constant current flowing through an irritable tissue is interrupted there is a stimulation (the "break" response) having all the characteristics (such as polar reversal) of stimulation by a polarization current. The polarized surfaces are those of the plasma membranes of the irritable elements, and the circuit is completed through the protoplasm and the interstitial fluids. An analogous activation on break of a constant current is also readily demonstrated in the passive iron wire, but only under somewhat special conditions. If we employ the usual arrangement, consisting of a battery of several storage cells connected through a key to a tube rheostat from which wires lead to the two passive wires in 70 v. per cent HNO_3 , we observe in closing the circuit, with a P.D. of 1 to 2 volts, the usual immediate activation of the cathodal wire followed by the automatic return to the passive state. On opening the circuit, however, nothing happens in the anodal wire, although it is now the cathode of the reversed polarization current, under these conditions the intensity and duration of this current are insufficient for activation. If we increase the polarizing potential, by moving the contact further along the rheostat, we increase correspondingly the resistance to the polarization current through the rheostat wire, and also increase the "anelectrotonic" stabilization of the anodal wire, both these conditions are unfavorable to activation by the polarization current and none occurs. The passive wire can however be made sufficiently "sensitive" to respond

to the polarization current by decreasing the concentration of the nitric acid, but if this is done another difficulty enters. In acid of less than 50 v per cent concentration the activated wire does not undergo spontaneous repassivation, but remains continually active. Hence if it is in the same circuit with another passive wire, the latter, being cathode, is automatically activated by the current of the couple formed by the two wires, unless other conditions are present to prevent. For example, the anodal wire may be kept passive by anodal polarization, with sufficient intensity of current, but although this wire at once becomes active when the polarizing current is broken, this activation is an effect not of the polarization current but of the current of the couple formed by the two wires. In general, when one of two passive wires, immersed in nitric acid and connected with each other through a low resistance, is activated directly, *e.g.* by scratching with glass or touching with zinc the other wire immediately becomes active also ("distance action" effect) ¹⁴

This difficulty can be removed by substituting a platinum electrode for one of the passive iron wires and making this electrode the cathode of the polarizing current. Wire and electrode are immersed in 25 v per cent (or weaker) HNO_3 . During the flow of the polarizing current the passive wire remains bright and unchanged, with oxygen bubbles forming on its surface. Then, on breaking the current, the wire, being now the cathode of the polarization current, is at once activated. A simple arrangement suitable for demonstration consists of two dry cells connected through a pole changer to the keys supporting the passive wire and the platinum electrode, connected also to these keys is a dial resistance forming an adjustable shunt. With a moderate resistance (of 1 to 100 ohms) in the shunt the iron wire (as anode) remains passive while the battery circuit is closed. On breaking the circuit the wire is instantly activated. The necessary conditions are that the polarization potential should be sufficient and that the resistance to the polarization current should not be too high. If the shunt resistance is too low the current through the nitric acid between the platinum and the wire does not polarize the latter sufficiently, while if it is too high the polarization current is too weak to be effective.

GENERAL DISCUSSION

It seems clear that the various resemblances of behavior between the passive iron wire and the irritable living system (cell or axon) are to be referred to one special structural feature common to both systems, namely the presence of a thin, impermeable, electrically polarizable and chemically alterable surface layer or film. In both systems some critical chemical (or electrochemical) reaction occurs when the polarization of this layer is altered sufficiently (in one direction) and maintained at the altered level for a certain time. During this time an electric current passes across the surface, the flow of this current is attended with chemical decomposition and structural change in the film, as a result the system as a whole is activated.

In the iron wire in strong nitric acid the essential chemical events associated with the transmission of an activation wave are a reduction followed immediately by an oxidation, the former reaction disrupts the film, the latter restores it. Two reactions, opposed in their chemical character and physical effect, are thus identifiable in this system, and the facts of electrical activation cannot be understood without taking both reactions into account. Cathodal reduction, on which activation depends, is resisted by the oxidative action of the surrounding nitric acid, this action increases with concentration, and correspondingly the required intensity and duration of the activating current also increase. A further constant condition is that any region of the iron surface as it becomes active becomes also anodal, the resulting anodal oxidation is an additional factor opposing activation and tending to bring the reaction to rest. Under certain conditions this local anodal action may be sufficiently intense to prevent the active state from spreading, thus small active areas made by scratching a passive wire are repaired automatically (*i.e.*, repassivated) without giving rise to activation waves.^{1 10 14} In general we must conceive the process of activation in strong acid as always occurring against the resistance of a counter-reaction, which develops coincidentally with the activation and tends to restore the passive state. This is why the local reaction of activation is automatically self-limiting, one electrochemical reaction reverses or repairs the changes

produced by the other. The contrasted character of anodal and cathodal reactions is obviously the basis of the law of polar activation in the passive iron system, as well as of the phenomena of interference and reinforcement, resembling electrotonus, mentioned at the beginning of this paper.

Conditions of a closely analogous kind appear to exist in the irritable living system, although we must be on our guard against pushing the analogy too far,¹ since every model, including a mathematical one, is at best an approximation, and certain inconsistencies are inevitable. Nevertheless, the general resemblances seem to imply that when we stimulate electrically the living irritable system (e.g., muscle cell or nerve axon), the critical electrically controlled reactions—which we picture as occurring in the thin polarized layer of protoplasm immediately inside the cell boundary—are similarly contrasted in their general chemical character at the regions where the current enters and leaves the cell. If we regard the cell surface as an electrode surface and the protoplasmic layer immediately inside this surface as the solution in contact with the electrode, we should expect, on general electrochemical principles, that a current (positive stream) passing from cell surface to protoplasm would have an oxidizing action on the adjacent protoplasmic molecules, while at the regions where the direction of current is reversed the electrochemical effect would be reducing.² From this point of view the general electrochemical conditions would be similar in the protoplasmic system and in the passive iron model, regions of reduction corresponding to those where activation is initiated, and regions of oxidation to those where activation is arrested and the resting state restored. The general physical conception of an activation wave, disregarding the details of the chemical changes involved, would thus be the same for both systems. Other characteristic physiological conditions, such as

²¹ Hill has recently issued a warning against this, and I am in sympathy with the general tenor of his remarks—with certain reservations as to detail (*cf.* Hill, *A. V.*,² p. 47).

²² The conditions in electrostenolysis are similar, oxidation occurring at the surface of the membrane facing the cathode, $\pm e$, where the positive stream passes from membrane to solution.

the importance of oxygen and of anodal polarization²³ in the recovery of nerve, lend support to this view

This is perhaps as much as can be said with any justification at present, and it would be premature to interpret the facts of nerve metabolism during activity, so far determined, in terms of the alternating reduction and oxidation of special compounds present in the surface film of the excited area

SUMMARY

1 The relation between the $E M F$ and the minimal duration of an activating current has been determined for passive iron wires in nitric acid under varying conditions of concentration of acid, duration of recovery period, and presence of surface-action compounds

2 The characteristic intensity-duration curves resemble those of irritable living tissues with moderate speeds of response to stimulation (with chronaxies of the order of 10 to 30 σ)

3 The intensity of the current required for activation, as well as its minimal effective duration for a given intensity, increases rapidly with increase in the concentration of HNO_3

4 The responsiveness of the iron wire to brief currents is low immediately after activation and returns progressively to the original level during the immediately following period, at first rapidly and then slowly, following a time curve resembling the corresponding curve of living tissues during the relative refractory period

5 Surface-active compounds decrease reversibly, to a degree dependent on concentration, the responsiveness of iron wires to brief currents

6 Conditions are described under which the iron wire is activated by the break of an already flowing constant current

²³ Woronzow, D S, *Arch ges Physiol*, 1924, 203, 300

THE COMBINATION OF DIVALENT MANGANESE WITH CERTAIN PROTEINS, AMINO ACIDS, AND RELATED COMPOUNDS*

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INTRODUCTION

The present work deals with the combination which takes place between manganous ions and certain proteins, amino acids, and related compounds. It is a continuation of similar work dealing with certain of the other inorganic elements which has been carried out in this laboratory (1). For the purpose of this discussion, the term, "metallic complex," refers to that class of chemical compounds which are formed (a) between a positively charged metallic ion and either a negatively charged inorganic or organic ion to produce (b) a compound which is soluble in aqueous solution in which (c) the activity of the metallic ion is reduced and (d) in which the charge carried by the compound so formed may be equal to, or, in most cases, is different from, that of the constituent metallic element either in sign or in magnitude. The wide occurrence of manganese in biological substances (2) makes this study of timely interest.

Bivalent manganese is known to form complex ions with cyanides and pyrophosphoric acid (3). It also forms a series of relatively stable coordination compounds with ammonia and with water (4). The resemblance of the electronic structure of manganous ion to that of ferric ion, which forms complex ions with certain proteins, amino acids, and related compounds (1), leads one to expect that, under suitable conditions, it, too, will form complex ions with these substances.

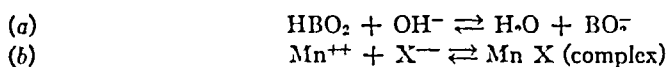
* Aided by a grant from The Chemical Foundation, Incorporated, and the Research Board of the University of California.

We are indebted to the Cyrus M. Warren Fund of the American Academy of Arts and Sciences for the loan of the potentiometer used in these experiments.

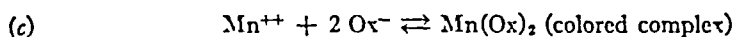
Distribution Experiments

Three procedures have been followed. The first consists in determining colorimetrically the relative concentration of manganous ions in the presence of the substance being tested for its ability to form complex ions. For this purpose the color which is produced in a chloroform solution of isonitrosoacetophenone, when shaken with an aqueous phase containing manganous ions and the test substance, was utilized. The aqueous solution was adjusted to pH 9.25 by means of a borate buffer. The two phases were shaken for 20 minutes. Tests showed that equilibrium was attained within 10 minutes. The color of the chloroform solution was then compared with the color of a similar solution treated under identical conditions with omission of the test substance from the system. In the event that a complex was formed between the manganous ions and the substance under test, a decrease in the color of the chloroform solution should be noted. Experiments showed that when light was passed through the chloroform solution of manganous oxime, Beer's law was not obeyed exactly, but to a degree which made the method of color comparison nevertheless usable for comparative purposes. The data obtained by the colorimetric method are semiquantitative and are therefore comparative rather than absolute.

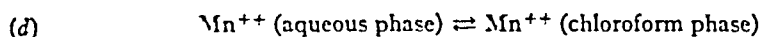
The validity of the distribution experiments depends upon sound theoretical considerations. In the aqueous phase, A , (Mn^{++} , HBO_2 , Na^+ , BO_2^- , X^{--} , where X^{--} represents the substance under test), the following equilibria exist



In the chloroform phase, C , (Mn^{++} , $\text{Mn}(\text{Ox})_2$, Ox^- , where Ox represents isonitrosoacetophenone), the significant equilibrium is



Between the two phases there exists the significant equilibrium in the case of manganous ions



At equilibrium the fugacity of Mn^{++} in phase A is equal to the fugacity of Mn^{++} in C . As the concentration of X^{--} in A is increased, equilibrium (b) is shifted in favor of MnX with the result that equilibrium (d) is shifted towards the left and this in turn leads to a like shift in equilibrium (c) .

The resulting decrease in the concentration of $\text{Mn}(\text{Ox})_2$ is measured

colorimetrically. The distribution ratio, in the *absence* of Ox^- and X^- (as in a solution of MnCl_2 distributed between water and chloroform)

$$[R] \approx \frac{\text{Mn}^{++} \text{ (aqueous phase)}}{\text{Mn}^{++} \text{ (chloroform phase)}}$$

must be very large

The color in the chloroform phase, when Ox^- and X^- are present, is due to a favorable distribution, not of Mn^{++} , but of the manganous oxime complex

$$R [\text{Mn}(\text{Ox})_2] = \frac{\text{Mn}(\text{Ox})_2 \text{ (aqueous phase)}}{\text{Mn}(\text{Ox})_2 \text{ (chloroform phase)}}$$

wherein the distribution ratio favors greatly the chloroform phase

The relation between the concentration of Mn^{++} in the borate buffer and the chloroform solution of oxime can be strictly linear only if (1) the extracting medium is constant and therefore always contains the oxime in large excess, and (2) a small amount only of the total Mn^{++} is removed from the aqueous phase, or (3) the complex in the chloroform layer is very stable and all of the Mn^{++} is extracted. In the latter case, the color intensity would not change with the concentration of oxime above a minimum value which it seems to do. It must therefore be assumed that in the chloroform layer, equilibrium (c) favors largely the dissociated form. To the extent that the manganous oxime complex is proportional to the Mn^{++} activity in the aqueous phase, Ox^- must be practically constant. The closely linear relation of "bound" Mn to the amount of X^- added, as will be shown later (see graphs) to be present in most instances, implies that the complex formed with X^- is very stable compared to the water or borate compounds of manganese which, in turn, are very stable compared to the oxime complex.

The following experiment was devised in order to determine whether the $\text{Mn}(\text{Ox})_2$ is dissociated to any appreciable extent. Orthophosphoric and malic acids were used as test substances. In the standard distribution procedure, the initial concentration of oxime in the chloroform layer was arbitrarily and progressively decreased in order to determine the minimum concentration of oxime which permitted the

detection of differences in the amount of colored complex formed in the chloroform phase due to differences in the concentration of the test substance in the aqueous phase. This is the concentration at which $\text{Mn}(\text{Ox})_2$ is dissociated to the extent that the influence of complex-forming ions in the aqueous phase competing for Mn^{++} is not detectable and therefore a measure of the relative degree of the dissociation of $\text{Mn}(\text{Ox})_2$. In the case of the above acids, the limiting concentration of oxime was obtained at 3.0 and 2.4 dilutions respectively of the chloroform phase. This implies that $\text{Mn}(\text{Ox})_2$ is appreciably dissociated by the competing equilibrium in the aqueous phase.

If MnX in the aqueous phase is largely undissociated in comparison to $\text{Mn}(\text{Ox})_2$ in the chloroform phase, then when the aqueous phase is reextracted with fresh portions of the oxime reagent, appreciable amounts of Mn^{++} should be successively extracted. The number of extractions in which Mn can be detected in the chloroform phase will be proportional to the amount of Mn "bound" in the form of MnX . Experiments in which orthophosphoric and malic acid were used as test substances showed that manganese could still be extracted with the oxime reagent after six and four extractions, respectively.

The presence of the borate buffer in the system for purposes of preventing pH changes presented the possibility that it might react with the polyhydric acids under test and thus prevent the latter substances from forming complexes with manganous ions. It is very probable that borates react with polyhydric alcohols and sugars. This leads to an increase in the acidity of the solution. However, the change in pH did not exceed 0.15 when mucic acid, in the concentrations used, was added to the buffer system. Moreover, $\text{Mn}(\text{OH})_2$ was precipitated from its solution in the buffer on addition of a sufficient amount of alkali. These facts, together with the fact that the amount of color obtained in the chloroform phase varied with the amount of the complex-forming substance added, indicated that the borate buffer was not a disturbing influence in the distribution experiments.

The results of the distribution experiments are represented graphically in Figs. 1 to 7. It is evident from Fig. 1 that the hydroxy-carboxylic acids (lactic, hydroxyacrylic, mandelic, glycollic) markedly affect the color of the chloroform phase whereas the unsubstituted acid,

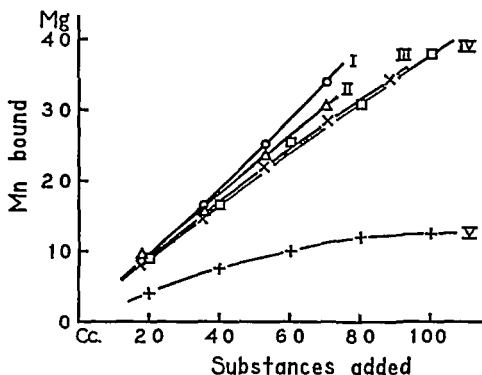


FIG 1 Total manganese present 5.5 mg total volume of aqueous phase 20 cc, volume of chloroform phase 15 cc, Curve I, 0.01 M hydracrylic acid, Curve II 0.01 M lactic acid, Curve III, 0.01 M mandelic acid Curve IV, 0.01 M glycollic acid Curve V, 0.01 M propionic acid

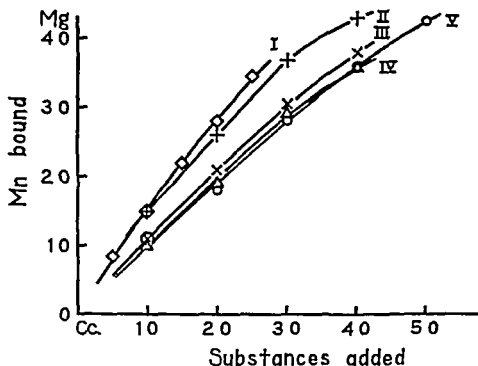


FIG 2 Total manganese present 5.5 mg total volume of aqueous phase 20 cc, volume of chloroform phase 15 cc, Curve I 0.01 M oxalic acid Curve II, 0.01 M malonic acid Curve III, 0.01 M succinic acid Curve IV, 0.01 M adipic acid, Curve V 0.01 M glutaric acid

COMBINATION OF DIVALENT MANGANESE

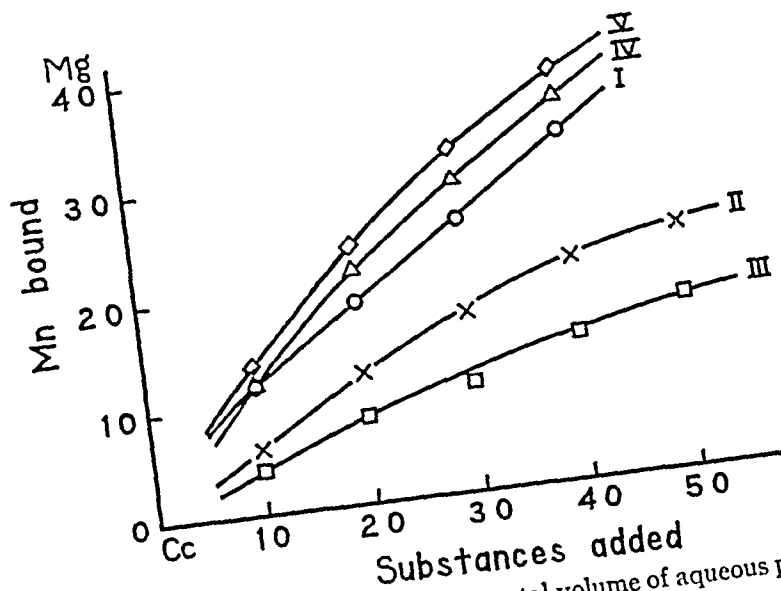


FIG 3 Total manganese present 5.5 mg, total volume of aqueous phase 20 cc, volume of chloroform phase 15 cc, Curve I, 0.005 M saccharic acid, Curve II, 0.005 M malic acid, Curve III, 0.005 M gluconic acid, Curve IV, 0.005 M tartaric acid, Curve V, 0.005 M citric acid

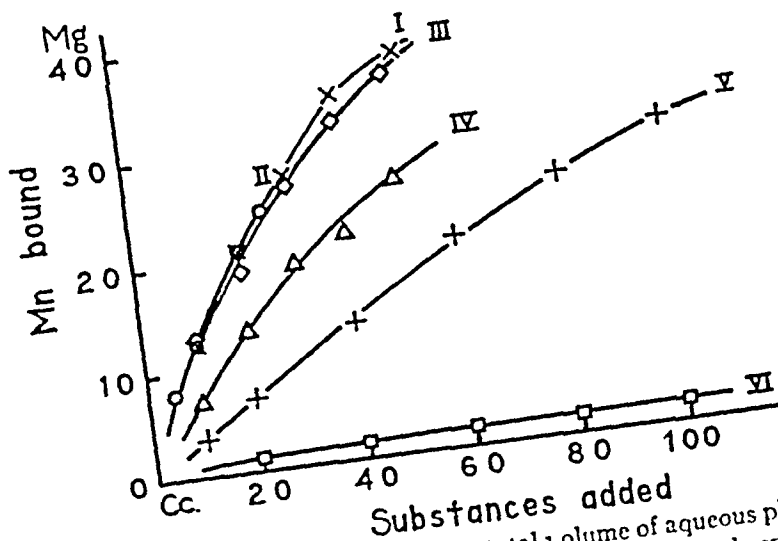


FIG 4 Total manganese present 5.5 mg, total volume of aqueous phase 20 cc, volume of chloroform phase 15 cc, Curve I, 0.0025 M glycerophosphoric acid, Curve II, 0.0025 M orthophosphoric acid, Curve III, 0.005 M metaphosphoric acid, Curve IV, 0.005 M sulfuric acid, Curve V, 0.01 M potassium cyanide, Curve VI, 0.01 M sodium chloride

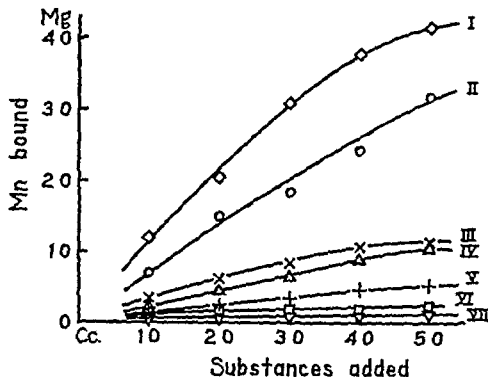


FIG 5 Total manganese present 5.5 mg total volume of aqueous phase 20 cc., volume of chloroform phase 15 cc., Curve I, 0.01 M aspartic acid, Curve II, 0.01 M glutamic acid, Curve III, 0.01 M arginine hydrochloride, Curve IV, 0.01 M glycine, Curve V, 0.01 M alanine, (also 0.01 M norleucine), Curve VI, 0.01 M ethyl amine hydrochloride, Curve VII, 0.01 M delta amino norvaleric acid (also 0.01 M gamma amino norvaleric acid)

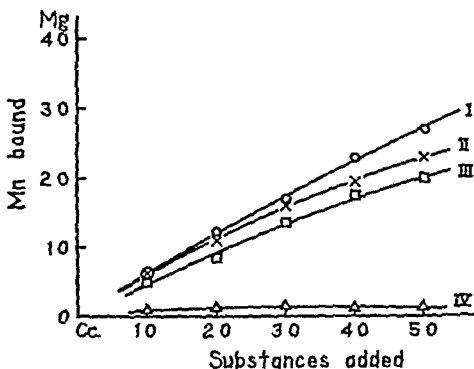


FIG 6 Total manganese present 5.5 mg, total volume of aqueous phase 20 cc., volume of chloroform phase 15 cc. Curve I 0.01 M lysine in 0.02 M sodium chloride, Curve II 0.01 M glycylglycine in 0.01 M sodium chloride, (also 0.01 M leucylglycylglycine, also 0.01 M leucylglycylglycylglycine), Curve III, 0.01 M leucylglycine (also 0.01 M tyrosine), Curve IV, 0.01 M glycine anhydride

propionic, exerts a much smaller influence. The dicarboxylic acids, oxalic, malonic, succinic, adipic, and glutaric (Fig 2), show a pronounced effect. The acids having the shorter chains are the more effective. When one or more hydroxy groups are present in the di- or tricarboxylic acid (Fig 3), the effect is usually greater than in the unsubstituted acid. Sulfuric acid, the several phosphoric acids, and potassium cyanide (Fig 4) also reduce the color of the chloroform phase. Aspartic and glutamic acid show a decided influence, while

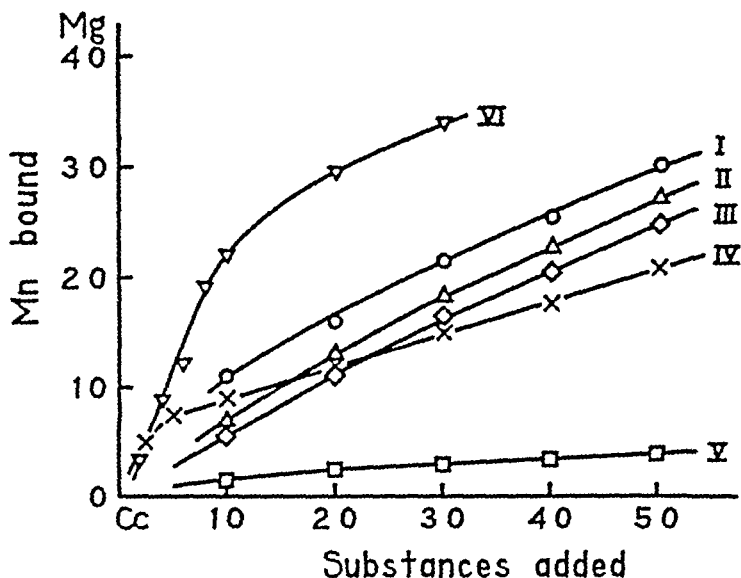


FIG 7 Total manganese present 5.5 mg, total volume of aqueous phase 20 cc, volume of chloroform phase 15 cc, Curve I, 0.0025 M thymus nucleic acid, Curve II, 1 per cent protamine sulfate, Curve III, 1 per cent edestin, Curve IV, 1 per cent gelatin, Curve V, 0.01 M proline, Curve VI, 1 per cent casein

the several amino acids listed under Fig 5 and ethylamine affect the color but little. The influence of the several polypeptides (Fig 6) is greater than their component amino acids, while glycylglycine shows no effect. Thymus nucleic acid, gelatin, edestin, and casein (Fig 7) show evidence of forming complexes with manganous ions.

Migration Experiments

Migration experiments were used as the second criterion to determine complex formation. For this purpose, the pH of the solution of

the test substance was adjusted a little more alkaline than finally desired, the manganous chloride was added, and any small amount of precipitate, if formed, was filtered off. Excess amount of manganous chloride beyond that required for the complex was avoided. The solution was subjected to migration in a 3 compartment cell using platinum wires as electrodes. Due to the pH of the solutions em-

TABLE I
Migration of Manganese in Solutions of Various Substances

Substance added	Original solution			Anode portion		Middle portion		Cathode portion	
	pH	Molality of substance tested	Manganese	pH	Manganese	pH	Manganese	pH	Manganese
			per cc		per cc		per cc		per cc
Oxalic acid	5.9	0.1	0.0721	2.6	0.0687	5.3	0.0744	7.7	0.0732
	9.1	0.1	0.185	4.4	0.198	9.7	0.165	10.8	0.188
Malonic acid	2.0	0.1	0.474	2.0	0.404	2.0	0.475	2.1	0.584
	8.0	0.1	0.900	5.0	0.912	7.6	0.904	9.2	0.848
Succinic acid	7.1	0.1	0.0480	5.6	0.0507	8.1	0.0475	11.4	0.0455
Tartaric acid	2.5	0.05	0.0805	2.2	0.0710	2.5	0.0802	2.7	0.0897
	7.8	0.05	0.0780	4.3	0.0833	8.4	0.0843	11.4	0.0663
Citric acid	7.9	0.05	0.0802	6.0	0.0918	8.7	0.0784	11.5	0.0705
Lactic acid	2.8	0.1	0.523	2.6	0.376	2.8	0.526	3.1	0.678
	6.7	0.1	0.0613	3.9	0.0561	7.2	0.0587	11.4	0.0629
Orthophosphoric acid	8.0	0.10	0.00419	6.7	0.00453	8.1	0.00420	11.3	0.00380
Aspartic acid	4.1	0.05	0.926	3.5	0.648	4.1	0.933	5.3	1.21
	9.0	0.05	0.0717	3.5	0.0721	10.1	0.0714	11.2	0.0711
Glutamic acid	4.6	0.05	0.900	4.2	0.646	4.6	0.946	5.5	1.103
	9.1	0.05	1.525	5.2	1.550	9.0	1.526	10.1	1.490
Glycerophosphoric acid	7.6	0.1	0.0313	6.5	0.0332	7.8	0.0306	11.1	0.0296
Casein	2.0	0.5*	0.123	1.9	0.109	2.0	0.115	2.2	0.144
	8.5	0.5*	0.0880	2.2	0.124	10.5	0.0915	11.4	0.0433

* Per cent

ployed, quantitative transference experiments could not be carried out. Manganese was estimated colorimetrically after removal of organic matter and subsequent wet oxidation to permanganate by means of ammonium persulfate in the presence of silver ion.

The data are presented in Table I. The experiments with lactic acid show that at pH 2.8, manganese is mostly cationic. At pH 6.7,

the relative amount of cationic manganese is decreased. In the case of oxalic and malonic acid, manganese migrates to the anode when the solutions are alkaline and to the cathode at the respective acidities given. At pH 7.1, manganese in the presence of succinic acid is

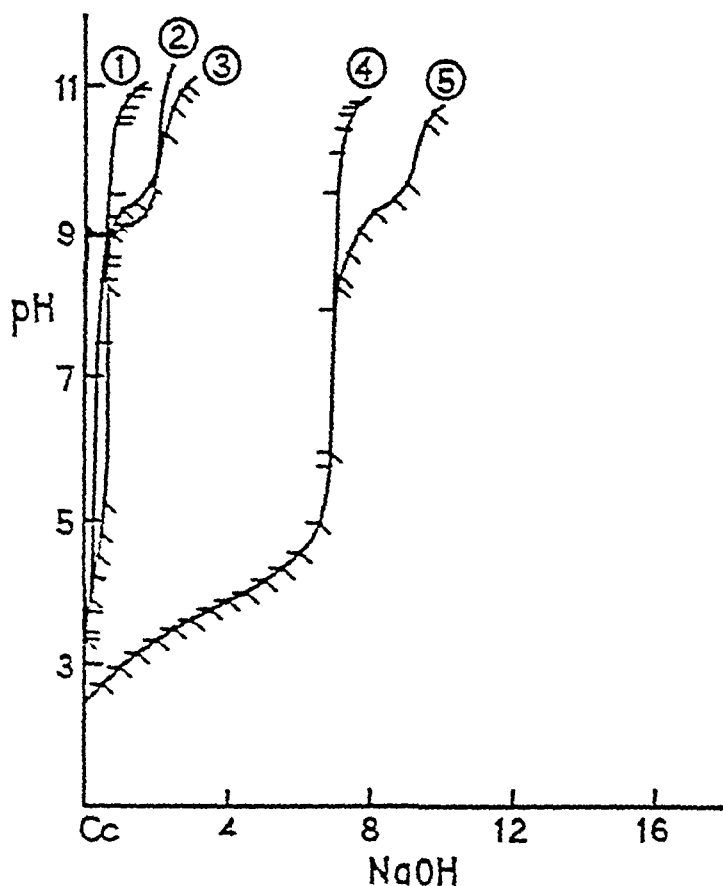


FIG. 8. Concentration of NaOH 0.1484 N, final total volume 22 cc., Curve (1), 10 cc. 8-hydroxy quinoline sulonic acid (saturated solution at 24°), Curve (2), 1 cc. 0.15 M MnCl_2 , Curve (3), 10 cc. 8-hydroxy quinoline sulonic acid solution, saturated at 24° - 1 cc. 0.15 M MnCl_2 , Curve (4), 5 cc. 0.2 M lactic acid, Curve (5), 5 cc. 0.2 M lactic acid - 1 cc. 0.15 M MnCl_2 . Temperature 24°C.

anionic. Manganese is anionic in the presence of citrates or tartrates at or above pH 7.8, while at about pH 2.5 in the presence of tartaric acid is cationic. In the presence of orthophosphoric acid manganese is anionic at pH 8.0. At pH 7.6 this is also the case when glycerophosphates are present in the solution. In solutions of glutamic and

aspartic acids at pH 4.6 and 4.1 respectively, manganese is cationic, whereas at about pH 9.0, it is anionic. The direction of migration of manganese in the presence of casein also depends on the pH of the solution.

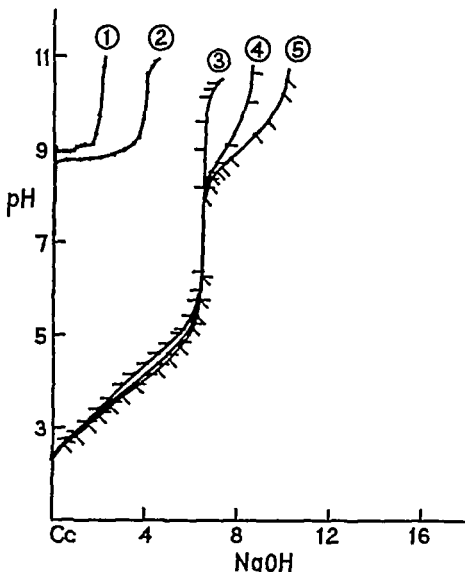


Fig. 9. Concentration of NaOH 0.1484 N final total volume 15 cc. Curve (1) 1 cc 0.15 M MnCl_2 ; Curve (2), 1 cc 0.3 M MnCl_2 ; Curve (3), 5 cc 0.10 M malic acid; Curve (4), 5 cc 0.1 M malic acid + 1 cc 0.15 M MnCl_2 ; Curve (5) 5 cc 0.1 M malic acid + 1 cc 0.3 M MnCl_2 . Temperature 24.

Anomalous Titration

The third criterion for complex formation was the anomalous behavior of certain organic acids when titrated with alkali in the presence of manganous ions. Similar work was carried out on magnesium by Zorkendorfer (5) and on iron and copper compounds by Smythe (6).

The titration curves were carried out with the aid of the glass electrode in a closed system after removal of oxygen by means of a constant stream of purified hydrogen. The titrations were carried out on portions of the same solution with and without manganous chloride

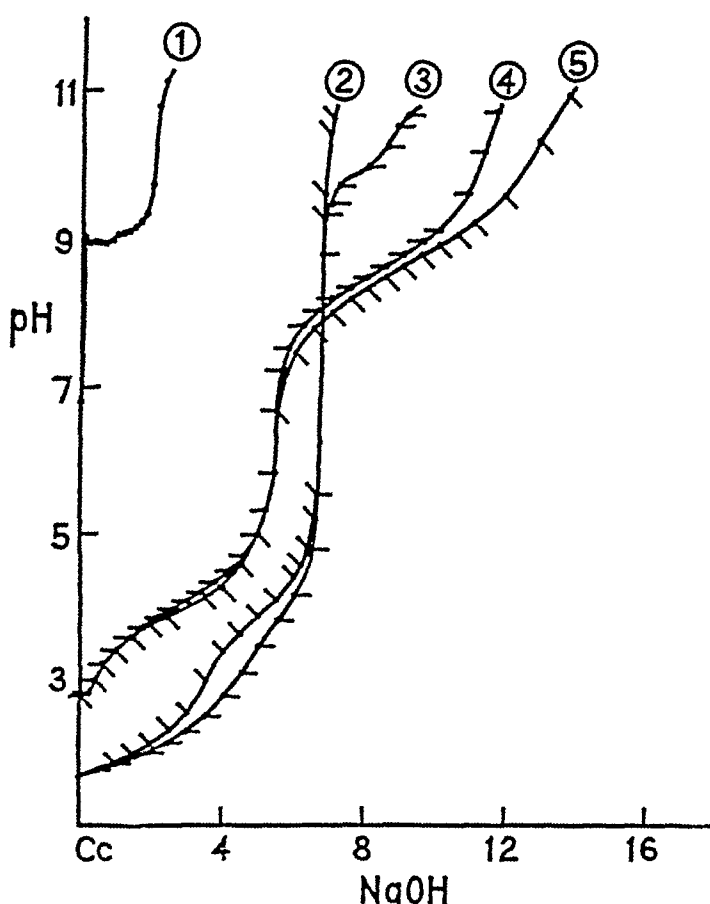


FIG. 10. Concentration of NaOH 0.1484 N , final total volume 22 cc, Curve (1), 1 cc 0.15 M $MnCl_2$, Curve (2), 5 cc 0.1 M oxalic acid, Curve (3), 5 cc 0.1 M oxalic acid + 1 cc 0.15 M $MnCl_2$, Curve (4), 10 cc saturated solution 2-amino-phenol-4-sulfonic acid at 24°, Curve (5), 10 cc saturated solution 2-amino-phenol-4-sulfonic acid + 1 cc 0.15 M $MnCl_2$. Temperature 24°C

The concentrations of the reagents were essentially the same as given by Smythe (6). The data are represented graphically in Figs. 8 to 12.

It will be noted that in most cases the mixtures of organic acids and manganous chloride are more acid than the organic acid itself. That

this effect is not more pronounced is due probably to the relative basicity of the manganous ions. The results are interpreted as indicating that the manganese containing complex is a stronger acid than the original organic acid added. In the case of lactic acid, the

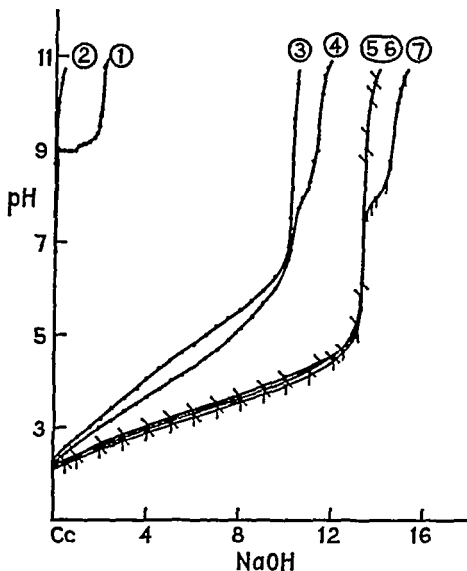


FIG. 11 Concentration of NaOH 0.1484 *N*, final total volume 22 cc. Curve (1) 1 cc 0.15 *M* $MnCl_2$, Curve (2), 1 cc. 0.3 *M* NaCl, Curve (3) 5 cc 0.1022 *M* citric acid, Curve (4) 5 cc 0.1022 *M* citric acid + 1 cc 0.15 *M* $MnCl_2$, Curve (5), (with spurs pointing upward), 5 cc 0.2 *M* tartaric acid + 1 cc 0.3 *M* NaCl, Curve (6) (with spurs pointing downward) 5 cc 0.2 *M* tartaric acid, Curve (7), 5 cc. 0.2 *M* tartaric acid + 1 cc 0.15 *M* $MnCl_2$. Temperature 24°

effect is shown only in regions of high alkalinity. Lactic acid does not prevent the precipitation of manganous hydroxide. The precipitation occurs when the amount of alkali added is equal to the sum of that required for the neutralization of lactic acid and manganous chloride

individually Malic acid likewise does not prevent the precipitation of manganous hydroxide In the case of 2-amino-phenol-4-sulfonic acid, the anomalous effect is more pronounced in the region of the second acid (enol) dissociation constant than in the first The anom-

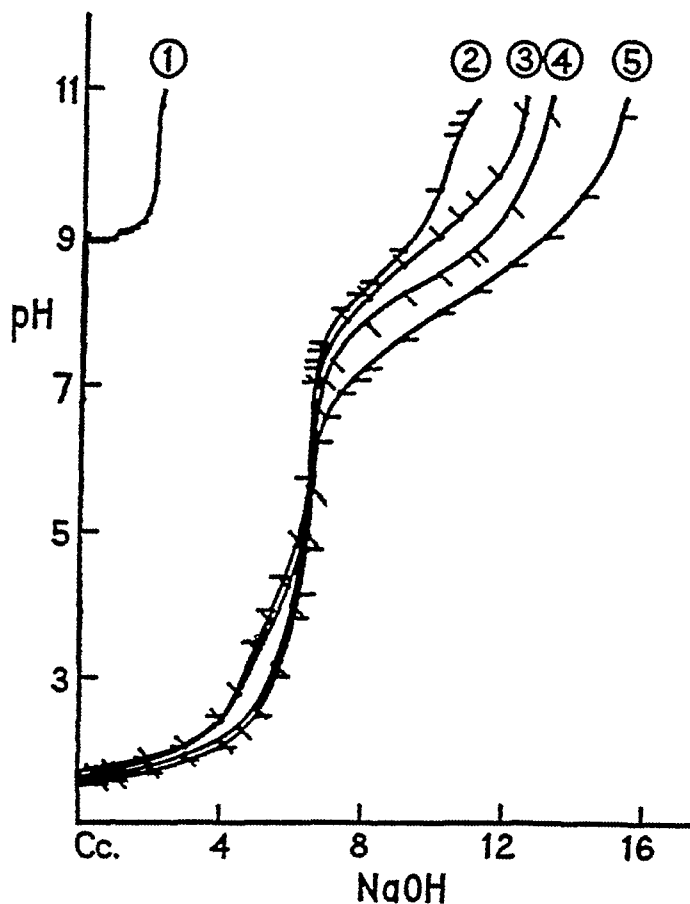


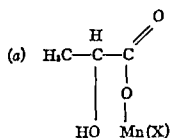
FIG 12 Concentration of NaOH 0.1484 N, final total volume 22 cc, Curve (1), 1 cc 0.15 M MnCl_2 , Curve (2), 5 cc 0.19 M phenolsulfonic acid, Curve (3), 5 cc 0.19 M phenolsulfonic acid + 1 cc 0.15 M MnCl_2 , Curve (4), 5 cc 0.19 M catecholsulfonic acid, Curve (5), 5 cc 0.19 M catecholsulfonic acid + 1 cc 0.15 M MnCl_2 Temperature 24°

alous effect is greater in the case of the dienolic (catecholsulfonic) acid than when the phenolsulfonic acid is present In the absence of oxygen, manganous hydroxide precipitates from a dilute solution of manganous chloride when the pH is adjusted to 9.0 In the presence of citrate, tartrate, and catecholsulfonate ions, precipitation does not

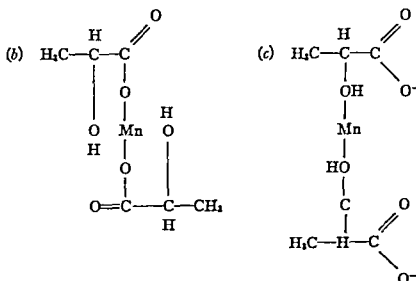
occur even at pH 10.6. When one considers that the solubility product of manganous hydroxide is of the order of 4×10^{-14} , it is evident that the activity of the manganous ions is markedly decreased by the presence of these ions. The curves for oxalic acid are complicated by the precipitation of manganous oxalate in the region of pH 2.5–9.7. Above pH 9.7, manganous hydroxide is formed. It is to be observed that the portion of the curve which corresponds to the second dissociation constant of oxalic acid lies in a more acid region when manganese is present than in its absence. If the formation of the complexes did not take place, it would be expected that manganous chloride like sodium chloride would decrease rather than increase the activity of the hydrogen ions of an acid like tartaric, however, this is not the case.

DISCUSSION

On the basis of the Latimer and Porter (7) theory, divalent manganese probably forms a complex with hydroxy acids of the type



where X represents any negative group. In this respect its behavior is like that of iron (1). Other structural possibilities are

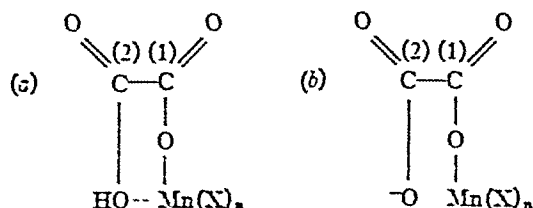


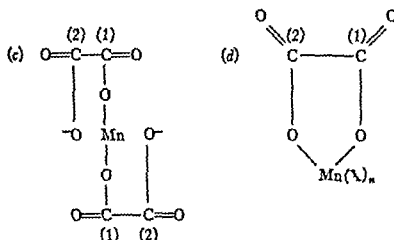
Since the migration experiments indicate that manganese is cationic in the presence of lactic acid at pH 6.7, formula (a) or (b) is to be preferred. Since the negative charge on the carboxyl group at this pH is greater than the residual negative charge on the hydroxyl group, it is not reasonable to assume that manganese would be attached to lactic acid exclusively by means of the attraction of the residual negative charge on the hydroxyl group in preference to that of the strongly negatively charged carboxyl group. If formula (c) were correct, then it would be expected that manganese would be anionic at pH 6.7.

If the monocarboxylic amino acids exist in the classical form, the residual charge on the nitrogen is -0.11 . Some complex formation with manganese should take place. If they exist wholly as zwitter ions, the residual charge is $+0.89$ and no effect would be shown. The available evidence indicates that there is an equilibrium between the two forms (8). Since it appears probable that under the conditions of the present experiments a portion of the amino acids tested exists in solution in the classical form, it is not at all unexpected to find that the monocarboxylic amino acids tested showed a slight tendency to form complexes with manganese.

In the case of oxalic, malonic, succinic, and glutaric acids, the data indicate that their relative ability to form complexes is in the order given. In each of these acids one carboxyl group furnishes the bond, the other furnishes the additional attraction. Within lower pH ranges, equimolar solutions at the same pH differ only in the concentration of the carboxyl group which furnished the bond. In solutions of pH 9.25 the first carboxyl group of oxalic and of malonic acid is completely ionized. The second dissociation constant of oxalic acid is greater than that of malonic acid. The strength of this carboxyl group determines the amount of additional attraction and hence the amount of manganese complex which is formed.

The following structural possibilities could exist in the case of the dicarboxylic acids

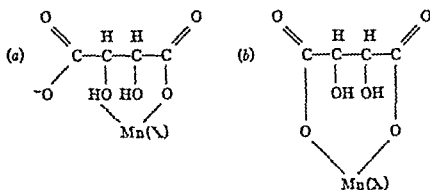


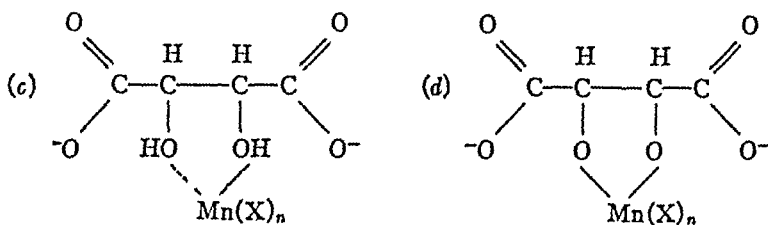


Formulas (a) and (b) differ only in the degree of dissociation of carboxyl group (2). In solutions whose pH is greater than 6.0, for formula (b) is the more plausible. Formula (c) is a special case of (b). Formula (d) represents a neutral molecule. It does not account for the fact that manganese is cationic at pH 5.9 and anionic at pH 9.1. The first three formulas are in agreement with the facts. If carboxyl group (2) ionizes, then manganese is anionic. If the pH of the solution is such as to repress the ionization of group (2), then the manganese which will migrate under the influence of a direct current is that which, on dissociation, will carry a positive charge.

The dicarboxylic amino acids react with manganous ions at pH 9 in a manner analogous to that of the unsubstituted dicarboxylic acids. The effect, as in the case of the latter compounds, depends upon the presence of the two free carboxyl groups in the molecule.

If, in addition to the carboxyl groups, one or more hydroxyl groups are present in the molecule to furnish additional attraction, conditions are particularly favorable for the formation of manganous complexes having a low degree of dissociation. This is borne out in the experiments with tartaric, saccharic, and citric acids. The following structural possibilities present themselves in the case of tartaric acid.





Formula (b) is not tenable since it represents a neutral molecule and does not explain the fact that at pH 7.8 manganese is anionic in the presence of tartaric acid. Due to the proximity of the two carboxyl groups, the hydroxyl groups of tartaric acid are more negative and more ionized than otherwise would be the case. Each hydroxyl group would not be expected to exhibit a negative charge comparable to that carried by an ionized carboxyl group, at least, not to the extent as to make formula (d) preferable to (a). The migration data do not enable a differentiation to be made between formulas (a) and (c). Formula (a) is to be preferred. It is more likely that manganese will be attached to the carboxyl group by a valence bond since this group is more negative than either of the hydroxyl groups of tartaric acid.

The object of the experiments which were carried out on the series of enol compounds was to determine whether the enol group, in conjunction with the sulfonic acid group which furnishes the bond, might yield the additional attraction which is necessary for the formation of manganous complexes. Calculation on the basis of residual valence of the oxygen atom in an undissociated enol group yields a value of -0.2 , the same as in the case of an hydroxyl group. If, however, the hydrogen atom of the enol group is dissociated as an ion, the residual valence of the oxygen atom is -1.2 . At higher pH values, the equilibrium between manganous ion and manganous complex should be shifted in favor of the complex. The titration curves of aminophenol- and of catecholsulfonic acids in the presence of manganous chloride and the migration data with phenolsulfonic acid confirm this prediction. The attraction for manganous ions is increased by the presence of 2 enol groups as in catecholsulfonic acid.

At pH 9.25, the di- and polypeptides studied form complexes with manganous ions to an appreciable extent, whereas, glycine anhydride does not. The difference in behavior is due to the presence of the free carboxyl group in each of the former compounds which furnishes the

bond The enol group of the peptid group furnishes the necessary additional attraction

It should be pointed out that this additional attraction is not merely a matter of charge In chloracetic acid the chlorine atom possesses a residual negative charge and yet this compound does not form complexes with manganous ions It is to be expected that the chlorine atom would be less effective than negative nitrogen or oxygen since it possesses a kernel charge of 7 in contrast to 5 and 6 respectively for the other two The greater positive kernel charge will offer greater repulsion to the approach of a positive charged ion This is in agreement with the fact that nitrogen and oxygen form onium compounds more readily than does chlorine

The general considerations given above can be applied to the complexes which are formed between proteins and manganous ions The assumption is made that the amount of manganese which combines with the free carboxyl groups in the protein molecule is the same irrespective of whether the amino acid which furnishes this group is in the free state or is combined as in the protein molecule This assumption was also made by Smythe and Schmidt (1) The additional attraction which is necessary for complex formation is probably furnished by the enol group of tyrosine (when present) and the enol groups of the peptid linkages The effect due to this attraction cannot be calculated quantitatively Qualitatively there is a correlation between the number of free carboxyl and phosphoric acid groups present in the three proteins studied and the amount of manganese which, under the conditions of the present experiments, was combined with the proteins The number of these free groups, as moles per 100 gm, are, in the case of casein, 0.174, in edestin, 0.073, and in gelatin, 0.041 According to the data represented in Fig. 7, the maximum amounts bound by these proteins are approximately in this ratio

SUMMARY

1 A study of the mode of combination which takes place between certain amino acids, proteins, various carboxylic acids, and certain sulfonic acids and manganous ions to form complexes is reported

2 Three criteria for complex formation were used (a) the equilibrium between the substance under test and manganous ions dissolved

in aqueous buffered solution and isonitrosoacetophenone dissolved in chloroform, (b) the electrophoretic migration of manganese in the presence of the test substance with varying pH, and (c) anomalous titration

3 The following classes of substances were found to possess the necessary groupings to form manganese complexes hydroxy-mono-carboxylic acids (lactic, gluconic), dicarboxylic acids (oxalic, malonic), hydroxy-, di- and tricarboxylic acids (citric, tartaric), dicarboxylic amino acids (aspartic, glutamic), certain inorganic acids (phosphoric, sulfuric), certain phosphoric acid-containing compounds (nucleic, glycerophosphoric), certain aromatic enol sulfonic acids (phenol-sulfonic, catecholsulfonic), and certain proteins (casein, edestin, gelatin)

4 A correlation between the amount of manganese bound by the several proteins and the free carboxyl and phosphoric acid groups has been made

5 An explanation based on the residual charge of certain atoms is advanced for the manner in which divalent manganese may be united by the compounds studied

We are indebted to Professor T D Stewart for valuable suggestions

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INCREASED PERMEABILITY TO WATER OF AGING UNFERTILIZED EGGS (*ARBACIA PUNCTULATA*)

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Unfertilized eggs of *Arbacia punctulata*, aged in sea water, manifested changes which indicated increasing water intake. These were progressive enlargement in sea water (Goldforb (1)), increased swelling and bursting rates in hypotonic sea water of the same osmotic pressure, temperature, and pH¹. Weber (2) noted that the plasmolysis rate of *Spirogyra* and of *Ranunculus* guard cells changed with age. Mestscherskaya (3) also observed that penetrability of dyes and acid in the oocytes of *Blatta* changed with age. The present study with aging *Arbacia* eggs demonstrates a progressive and marked increase in permeability to water.

Procedure

The experiments were performed at the Marine Biological Laboratory at Woods Hole with the assistance of Victor Schechter in 1930, and of Milton Landowne in 1931 and 1932. The *Arbacia* were received directly from the collecting boat. The eggs of each female, if in good condition were separately strained and washed in 200 cc sea water. Aliquot samples were placed in flat bowls each with 150 cc sea water. The sea water was changed twice daily. Sea water was collected near high tide filtered and stored before using. The pH was 8.3. The temperature in the bowls ranged from 16.8–22.4°C but varied within 2° during an experiment.

Permeability rate was determined by the method of Lucké and McCutcheon (4–10). A dish containing 15 cc 60 per cent sea water was placed in an outer dish. Ice water was added to the latter to maintain a temperature of 21° or 21.5°C with a variation within 0.4°. frequently within 0.2°. A 0.1 cc sample of eggs was transferred to the inner dish quickly and thoroughly stirred. The diameters of two or three adjoining spherical eggs were measured each minute for 5 to 7 minutes. Measurements were made with water immersion objective

¹ To be reported later

and ocular micrometer, magnification 660 x, or, with a dry objective and filar micrometer, 260 x. Measurements were consistent within 0.4μ . The probable error was $\pm 0.11\mu$.

This procedure was repeated until about twenty eggs were measured at each age. The average size each minute was plotted, the curve drawn, and the slope determined by the tangent at the 3rd minute. The permeability rate or $K =$

$$\frac{dv/dt}{S(P-P_{ex})}, \text{ in which}$$

$\frac{dv}{dt}$ = rate of change in volume or rate of water intake,

S = surface area,

P = osmotic pressure of interior of cell,

P_{ex} = osmotic pressure of the solution

The permeability rate measures the amount of water in μ^3 , passing through unit surface (μ^2) per minute, at unit osmotic pressure difference, at 21°C , pH 8.3

The condition of the eggs at successive ages was ascertained by the per cent cleaving after insemination with freshly prepared 1 per cent suspension of fresh dry sperm.

Increased Swelling with Age

Before calculating the permeability rates, it was noted that swelling increased with age. Experiment E, Table I, illustrates this phenomenon. Fourteen to twenty-one eggs were measured in 60 per cent sea water at each age, viz., $1\frac{1}{2}$, 5, 9, 24, 32, and 47 hours after shedding. The average size at the end of 5 minutes, or V_5 , increased with age from 276,200 to 289,300 μ^3 . The increase was 13,100 μ^3 or 4.7 per cent. But V_5 is not an accurate index of swelling. For it was demonstrated (Goldforb (1)) that V_0 , i.e. initial size, is not constant, but undergoes a cyclical change with age. $V_5 - V_0$ is therefore a more accurate index of swelling. This $V_5 - V_0$ increased with age from 67,900 to 76,200 μ^3 , i.e., 8,300 μ^3 or 12.2 per cent (Table I).

The rate of swelling correspondingly increased with age, i.e., from 13,600 to 15,200 μ^3 per minute (Table I).

Repeated experiments gave similar results. Under the same external conditions, the amount and rate of swelling increased with age.

Variation in Permeability Rates of Freshly Shed Eggs from Different Females

The permeability rates were calculated for freshly shed eggs from each of nine females. These rates varied from 0.078 to 0.124, with

an average of 0.106 ± 0.0034 . This variation is somewhat greater than recorded by Lucké and McCutcheon (4-10) for *Arbacia* eggs under similar conditions.

This variation may not be attributed to differences in physiologic condition, for the eggs ranged from 98 to 100 per cent normal cleavage, and differences were not correlated with permeability. Nor was variation in permeability correlated with age. The data suggest, however, that permeability may be highly correlated with initial average size of the eggs. The smallest averages ranged from 177,700 to 187,500 μ^3 . It is precisely these eggs that had the lowest permea-

TABLE I

Experiment E Shows Increasing Amount and Rate of Swelling with Age

Eggs from one female measured in 60 per cent sea water, pH 8.3, 21.5°C, for 5 minutes at each age

Age	$V \times 100\mu^3$	$V_s \times 100\mu^3$	Amount of swelling $(V - V_s) \times 100\mu^3$	Rate of swelling $\times 100\mu^3$	No. of eggs	Cleavage
hrs						per cent
1½	2033	2762	679	136	16	100
5	2073	2774	701	140	15	99
9	2140	2840	700	140	19	97
24	2143	2885	742	148	21	86
32	2144	2893	749	150	18	74
47	2126	2888	762	152	14	27
Increased swelling		13100	8300			
Increased percentage		4.7	12.2			

bility rates, *viz*, 0.078 to 0.094. The largest average sizes were 200,300 and 208,300 μ^3 . These eggs had the highest rates, *viz*, 0.123 and 0.124. Eggs of intermediate average sizes had intermediate permeability rates. There were, however, two apparent exceptions.

Increasing Permeability with Age

Whatever the initial permeability rate, it increased with age. Fig 1 illustrates this increase. One female was selected whose eggs were in good physiologic condition. Fifteen to twenty-one eggs were measured in 60 per cent sea water at 21°C, at each age, *viz*, 1, 3, 6, 9,

12, 19, 22, 25, 28, 34, and 37 hours after shedding Permeability rates (indicated by + in Fig 1) increased with age from 0.089 to 0.134, or 51 per cent

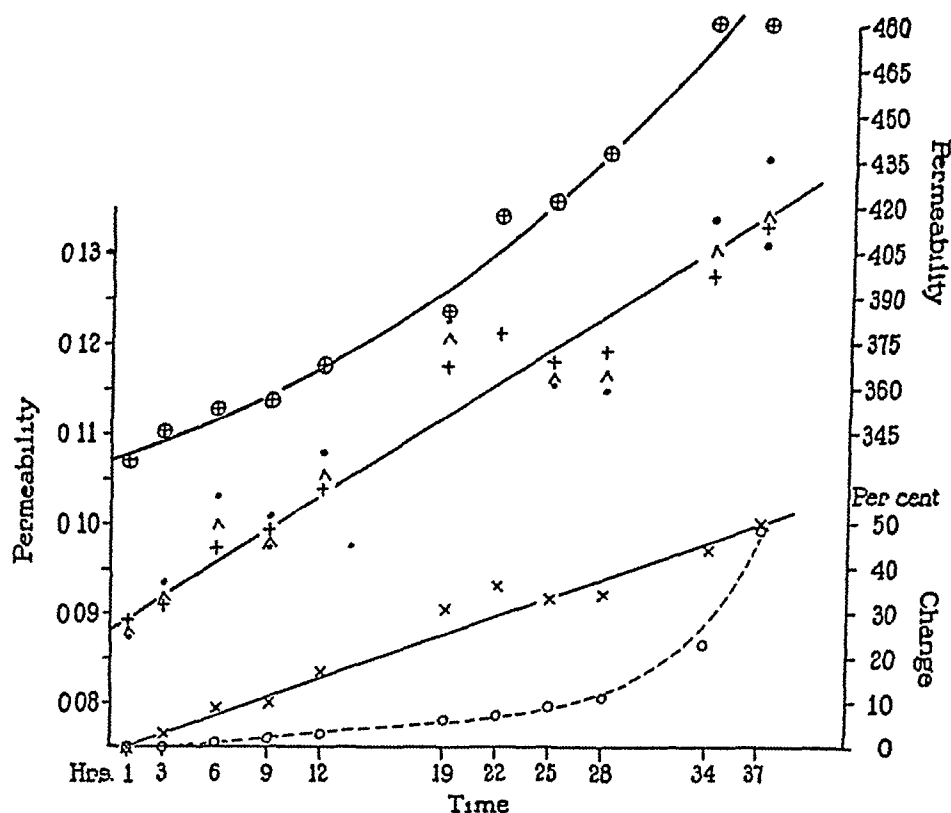


FIG 1 Experiment P Permeability increased with age at approximately constant rate Increasing permeability is not correlated with degree of injury Fifteen to twenty-one, average eighteen eggs measured each age in 60 per cent sea water at 21°C Maximum extrapolated V_0 at each age Permeability determined by the graphic method of Lucké, rates on left side of Fig 1

+ = approved permeability rates, believed most accurate, • = other possible rates, ^ = averaged rates, x = percentage increase in permeability, o = percentage injury, *ie*, decreased cleavage, ⊕ = permeability rates as determined by integration of Northrop's equation, on right side of Fig 1

All factors in the permeability equation may be determined with considerable accuracy, except the initial size of the eggs, or V_0 . This V_0 is obtained by extrapolation But it was frequently possible to draw the curve to different V_0 's, and the resulting permeability rate

may be correspondingly altered. In Fig 1, the V , selected for each age, was the maximum permitted by the curve. This maximum was essentially the same as the average size of fifty control eggs of the same age. The permeability rates, based on these V 's, are believed to be as accurate as the method permits. Such permeabilities are termed approved rates indicated by +. When, however, the curve of swelling was redrawn to other possible V 's and the permeability rates recalculated, other possible permeability rates were obtained. These are indicated by dots. They differed from the approved rates by 0.0015 to 0.007 units. At latest ages the variation was greater. Fig 1 also includes the average rates, at each age, represented by the symbol Δ .

The approved rates increased with age from 0.089 to 0.134. The other possible rates gave almost parallel increases, viz, from 0.088 to 0.141. The averages increased the same as the approved rates, viz, 0.088 to 0.135. The range of rates, each age, increased similarly, i.e., from 0.088 to 0.089 when 1 hour old, to 0.131 and 0.141 when 37 hours old. There can be no doubt that in this experiment, under the given conditions, there was a significant increase in permeability as the eggs aged.

Progressive Series of Small Increments—From Fig 1 it is evident that permeability did not remain at the same initial rate during early and intermediate ages, that it did not increase by a steep bend of the curve at late ages only. The data may be interpreted as an increase in four major steps, viz, an initial permeability about 0.090 (between 1 and 3 hours), a second level about 0.100 (between 6 and 12 hours), a third level at 0.119 (between 19 and 34 hours), and a fourth level 0.134 (at 37 hours). These four steps are not only quite arbitrary, but it is difficult to conceive of factors that might give rise to four such increments at these ages. In our other studies of size, viscosity, swelling, bursting, and oxidation of aging eggs, there is no indication of successive waves of change.

The alternative interpretation is that permeability increased in a linear series of many small increments. In support of this interpretation are the following. A line may be drawn through, or reasonably close to, successive permeabilities. This line is shown in Fig 1. The rate of increase is very slow, viz, 0.0012 units per hour. The possible permea-

bilities, at a given age, may vary between 0.0015 and 0.007 units (more at latest ages). Hence it is not possible to demonstrate an increase during short intervals. One must either examine longer intervals, *i.e.* at least 6 hours apart, or note the trend of the whole curve. Successive intervals 6 or more hours apart show not only progressive increases, but these increases are approximately equal (except between 22 and 28 hours). Similar results are obtained whether average, or range of permeabilities are used as a basis of comparison. While the results especially at late ages are somewhat irregular, it is believed that the line closely approximates the actual increase, *viz.*, by a linear series of small increments.

The data were recalculated by integration of Northrop's equation. The resultant values are represented in Fig. 1 by the symbol \oplus . The permeability rates increased with age from 0.00336 to 0.00480. This is an increase of 42.4 per cent. The resultant curve like that obtained by the graphic method of Lucké and McCutcheon shows a linear series of small approximately equal increments during early and intermediate ages. During late ages the increases are greater.

Is Increased Permeability Correlated with Degree of Injury?—To determine such correlation, permeabilities are represented in the lower part of Fig. 1 as percentage increase. Injury is indicated by percentage decrease in cleavage. It will be observed that the total change in injury and in permeability was the same, *viz.*, 50 per cent. The curves, however, are quite different. Permeability increased at essentially a constant rate throughout the experiment, injury, on the other hand, increased at differential rates.

During the first 9 hours cleavage was regular and the percentage decreased only 2 per cent. This is probably not significant. Permeability increased 12 per cent. Permeability therefore increased when there was little or no injury.

Between 9 and 28 hours, there was progressive injury. Cleavage decreased from 2 to 11 per cent. After 22 hours eggs cleaved irregularly. Permeability increased 26 per cent. But this increase was at the same rate as during the first 9 hours.

Between 28 and 37 hours the eggs deteriorated very rapidly. Cleavage decreased 38 per cent (*i.e.*, from 11 to 49 per cent). Cleavage was more and more irregular. Yet permeability increased during this

rapid deterioration at approximately the same rate as during all preceding ages

The increase in permeability therefore is not commensurate with the degree of injury. It increased at approximately constant rate, when injury was nil, moderate, or extensive

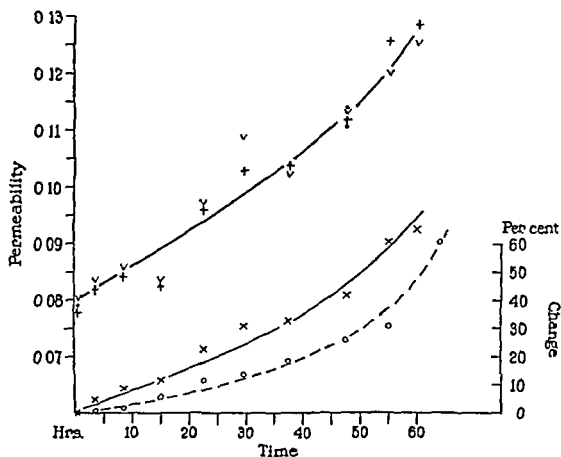


FIG 2 Experiment L Permeability increased at approximately same rate during early and intermediate ages accelerated rates during late ages No correlation with degree of injury Thirteen to eighteen, average sixteen eggs measured each age in 60 per cent sea water at 20° Maximum extrapolated V 's approximated control size each age Symbols same as in Fig 1

Experiment L

The experiment was repeated with similar results Measurements began earlier ($\frac{1}{2}$ hour) and extended over a longer time (60 hours) Thirteen to eighteen (average sixteen) eggs were measured each age The results are given in Fig 2 They may be summarized as follows

Permeability increased with age from 0.078 to 0.129, or 62 per cent The approved rates (represented by +) were calculated on the basis

of maximum V_o 's, which corresponded closely to the size of fifty control eggs at each age. The other possible permeabilities are represented by dots. Parallel increases occurred whether approved, average, or range of permeabilities were used as criteria of change.

The increase possibly occurred in four major steps, *viz*, from 0.085 to 0.096, 0.104, 0.112, and 0.126. But for reasons discussed in Experiment P, it is much more probable that it occurred in a linear series of many small and approximately equal increments represented by the uppermost line. This line passes through or reasonably close to the approved or average permeabilities. The rate of increase was slower than in any of the other eight experiments, *viz*, 0.0008 units per hour. Therefore intervals at least 9 hours apart are necessary to demonstrate an increase. At successive 9 hour intervals, permeability increased at approximately equal rates during early and intermediate ages, and faster during latest ages. It is therefore believed that permeability increased, as in Experiment P, by a linear series of small nearly equal increments, and that the increase began at the earliest age.

Permeability was not directly correlated with degree of injury. It increased during the first 7 to 9 hours with little or no injury. It increased at the same rate, between 9 and 47 hours, while eggs were deteriorating rapidly. It increased faster during the exceedingly rapid and intense deterioration, *viz*, between 47 to 60 hours.

The results in these two experiments are in close agreement.

Initial Size Factor

Various methods were used in determining the initial size, or V_o . Since control eggs swelled to 5.7 per cent during the first 25 hours and shrank thereafter to 9.7 per cent (Goldforb (1)) it was assumed that in the permeability experiments also, the control eggs changed in size in a similar cyclical manner, within approximately the same limits, in the same time. When several V_o 's were possible that one was selected which conformed to this cyclical trend. Permeabilities were calculated on this basis, in four experiments, namely, D, E, P₂, and P₃.

In two experiments, *viz* D and I, all the approved successive V_o 's were averaged, and the permeabilities recalculated on the basis of this constant average V_o . This method gave irregular increments

In three experiments, *viz*, H, I, and K, fifty control eggs were measured at each age immediately after the experimental eggs. Such V_0 was chosen as closely approximated the corresponding average size of control eggs of the same age.

In Experiment L the maximum V_0 , permitted by the curve, was selected. These maxima closely approximated the corresponding control size (of fifty eggs) at each age.

In two experiments, P and L, the minimal V_0 's were selected each age, but because the curves were almost tangent to the zero ordinate and because the V_0 's were so much smaller than the control sizes, the resulting permeabilities were discarded.

Whatever method was used the resulting permeabilities increased with age in every experiment. When calculated by different methods (except by constant average V), not only was the total increase very similar, but also the increments at successive ages. The results in the nine experiments are in reasonably close agreement. It is therefore believed that the values approximate very closely the actual increases in permeability with age.

Summary of Seven Other Experiments

Seven earlier experiments were performed under similar conditions. Their duration varied from 24 to 60 hours. Permeability increased with age in all of them. The increases (including Experiments P and L), ranged from 29 per cent (in 32 hours, Experiment E) to 69 per cent (in 44 hours, Experiment I (Figs 1 to 8)).

In Experiment E, the approved permeability rates increased with age as follows: 0.124, 0.134, 0.143, 0.155, and 0.158. The increase was 29 per cent in 32 hours. The curve is slightly convex (Fig 3). When recalculated by integration of Northrop's equation a similar progressive increase occurred with age, see uppermost curve. This is also convex at late ages.

In Experiment H (Fig 4) the results are essentially the same as in Fig 3. The curve is slightly convex. The increase in 48 hours was 40 per cent.

Experiment D (Fig 5) was our first experiment. Permeabilities were based upon extrapolated and upon an averaged constant V . The permeabilities by the latter method, represented by the symbol C

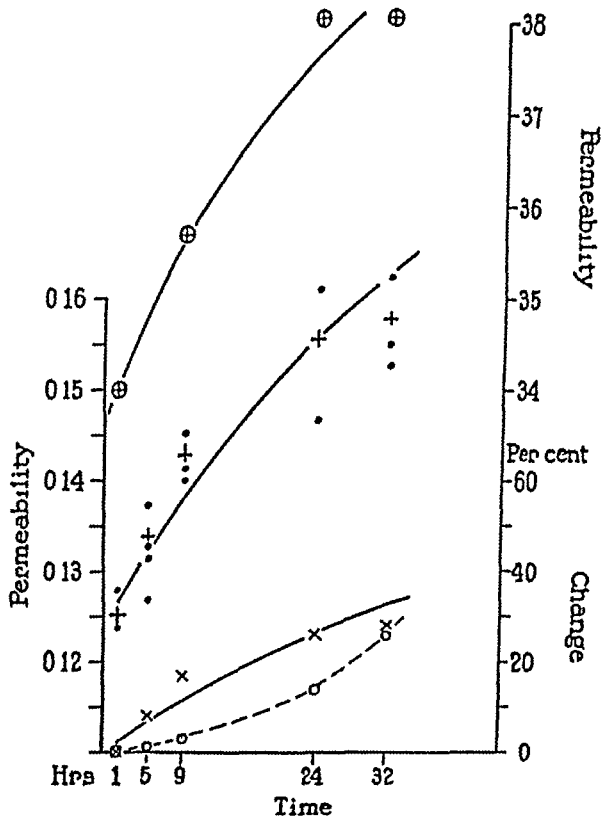


FIG 3 Experiment E Fifteen to twenty-one, average eighteen eggs each age, in 60 per cent sea water at 21°C Extrapolated cyclical V_o 's

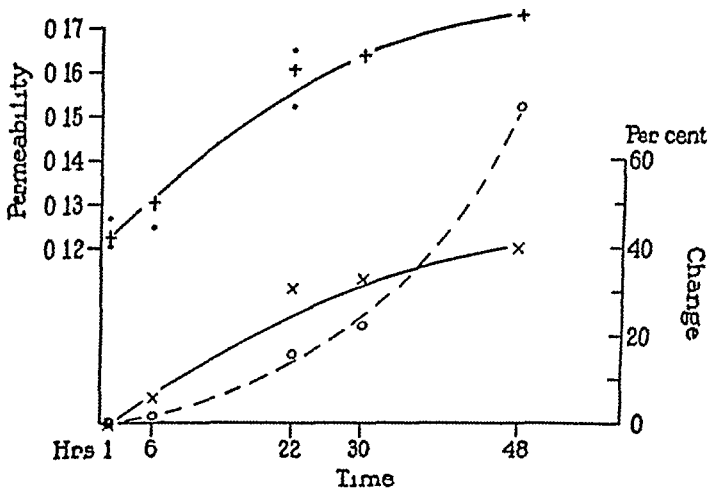


FIG 4 Experiment H Ten to fifteen, average thirteen eggs each age, in 60 per cent sea water at 21°C V_o 's approximated control sizes

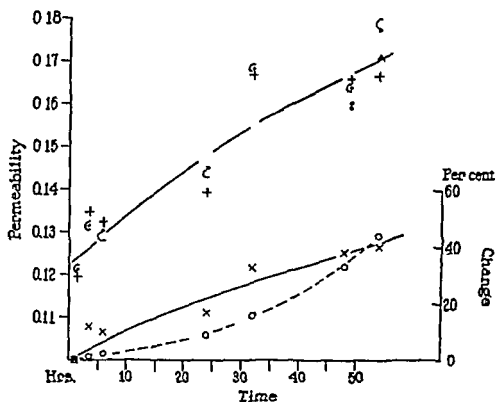


FIG 5 Experiment D Twelve to fourteen eggs each age, in 50 per cent sea water at 21° Permeability calculated with extrapolated V 's and recalculated with averaged V 's Latter permeabilities are represented by C

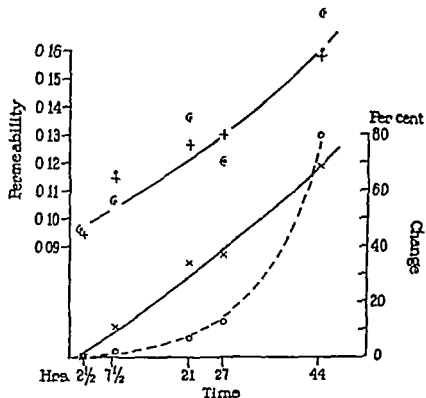


FIG 6 Experiment I Six to twelve average ten eggs each age, in 60 per cent sea water at 21° Extrapolated V 's approximated control sizes Permeabilities recalculated with constant averaged V 's, represented by C

gave irregular increments The curve of approved values is slightly convex The increase in 54 hours was 40 per cent

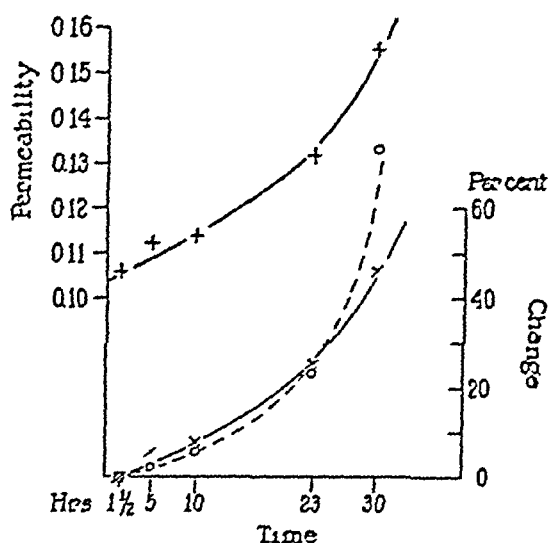


FIG 7 Experiment K Six to fourteen, average twelve eggs each age in 80 per cent sea water at 21° Extrapolated V_o 's approximated control sizes

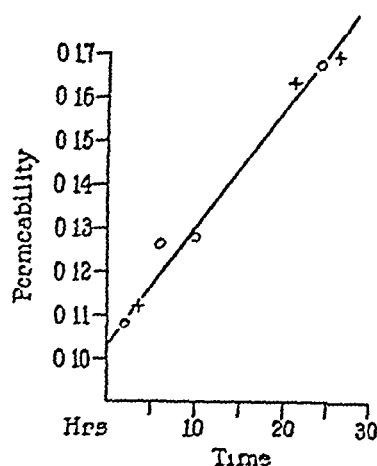


FIG 8 Experiments P_1 and P_2 Seven to twelve, average ten eggs each age, in 60 per cent sea water at 21° Extrapolated V_o 's approximated control sizes Permeability rates in Experiment P_1 represented by O, in P_2 by +

In Experiment I (Fig 6) the approved permeability rates were 0.094, 0.114, 0.126, 0.129, and 0.158 respectively The increase began

at the earliest ages and continued essentially at a constant rate throughout the experiment. The increase in 44 hours was 69 per cent. The permeability rates recalculated by the method of a constant average V_0 gave irregular increments. Recalculated by Northrop's equation the permeability rate increased from 0.00392 to 0.00539 or 37.5 per cent.

In Experiment K (Fig. 7) the permeability curve is slightly concave. The increase in 30 hours was 46 per cent.

In Experiments P₂ and P₃ permeability was determined at a few ages only (Fig. 8). They suggest linear equal increments.

These nine experiments gave substantially similar results. In all of them permeability increased with age by a linear series of small increments. During early and intermediate ages the increases were equal or nearly equal. At late ages the increases were equal in four experiments, greater in two experiments, and slightly lower in three experiments. For reasons to be given later it is probable that the actual increases were progressively greater at late ages in all experiments.

Permeability and Injury—As in Experiment P, increased permeability was not directly correlated with increased injury. See Figs. 3 and 6, also Figs. 4 and 5. During the earliest 6 to 9 hours there was little or no detectable injury, yet permeability increased. During intermediate ages there was progressive injury, yet permeability increased at the same rate as before injury. During late ages injury was rapidly accelerated, permeability also increased at somewhat greater rate.

Permeability during Late Ages

Beginning about the 24th hour, eggs deteriorated rapidly. Cleavage was progressively more irregular, and greater numbers did not cleave. When unseminated they may appear normal. That they were injured, however, was shown by atypic swelling during the test. These atypic eggs fall into the following groups:

1. Some eggs swelled distinctly faster than any of the other eggs of the same sample, and cytolized during the test, *i.e.*, during the first 5 minutes.

2. Other eggs also swelled much faster but for 2 to 3 minutes only. They ceased swelling by the 4th or 5th minute. No swelling occurred during the following 18 minutes under observation.

3 Some eggs either did not swell at all or swelled before they could be measured, *i e*, before the first half minute

4 Some eggs ejected a small pellicle of protoplasm, 1.5 to 3.75 μ diameter. About 2 minutes thereafter the egg, except for the pellicle, appeared normal in shape and color. Swelling during and immediately after pellicle formation was atypic.

The numbers of these atypic eggs increased during late ages. Their permeability rates were evidently greater than the other eggs from the same sample. But accurate determination of their rates was exceedingly difficult. Hence they were excluded. *The rates for the remaining eggs therefore undervalue the actual average permeability rates, at late ages*

Increased Permeability and Injury

Injury by heat (4-6, 9), hydrogen ion concentration (6, 14-16), anesthetics (17), or other means (18-22) markedly increased permeability. To determine whether, in our experiments, injury was induced by the experimental procedure, eggs were subjected at successive ages to 60 per cent and then to 100 per cent sea water for 7 minutes each at 22°C. Fifty control and fifty deswelled eggs were then measured, others inseminated. The deswelled eggs were approximately the same size (2 per cent larger) than the controls. This slightly larger size of deswelled eggs is in accord with the findings of Lucké and McCutcheon (4, 5) for freshly shed eggs. It is uncertain whether this indicates injury. The percentage and regularity of cleavage was the same for the deswelled and for the corresponding control eggs until about the 20th hour. After about 24 hours the deswelled eggs manifested injury which increased with age. Fewer eggs cleaved and were decidedly more irregular than the corresponding controls. In 80 per cent sea water injury was postponed to about the 28th hour. It is probable, therefore, that the experimental conditions induced no injury prior to the 24th hour. Injury was induced, however, after this age.

Aging in sea water at 21°C gave rise to no injury between 0 and 9 hours. Injury was evidenced between *ca* 9 and 23 hours. It increased with further aging. After *ca* 24 hours injury increased at

very rapid rates, and the eggs were increasingly susceptible to injury by the experimental procedure

Permeability also increased. But the curves of permeability and of injury were not parallel. Permeability increased during early and intermediate ages by approximately equal increments, whereas the injury curve showed no change during early ages and progressively larger increments during intermediate ages. At late ages the faster swelling atypic eggs were excluded. The actual rates, therefore, were greater during late ages, than shown in Figs 1 to 7. If these atypic eggs could have been included the curves of permeability would all be concave. In other words, extensive injury at late ages was accompanied by correspondingly increased permeabilities. But during early and intermediate ages (*i.e.*, before atypic eggs appear) the permeability increased apparently independently of the degree of injury.

It may be noted that swelling rate, in a given hypotonic sea water, increased markedly with age, and that this increase was even more clearly independent of the degree of injury.

Northrop (23) has urged that the graphic method of Lucké and McCutcheon for determining permeability is not satisfactory, for it depends too much on the swelling rate at a selected time (*i.e.*, the third minute). He believes that integrating the following equation is more accurate, *viz.*,

$$C_1(36\pi)^{1/2}P_0V_0t = V^{1/2} \left[\frac{1}{2} \ln \frac{V^{1/2} + (VV)^{1/2} + V^{1/2}}{(V^{1/2} - V^{1/2})^2} - \sqrt{3} \tan^{-1} \frac{2V^{1/2} + V^{1/2}}{\sqrt{3} V^{1/2}} \right]_{V_1=0}^V$$

Lucké, Hartline, and McCutcheon (4, 7, 12), however, compared the results obtained by the two methods and found them quite similar. We also recalculated the data in three experiments by the two methods. In all three experiments both methods gave a progressive series of small essentially equal increments during early and intermediate ages. During late ages the increases were greater in Figs 1 and 4, and slightly less in Fig 3. The two curves of permeability in each experiment were essentially parallel during early and intermediate ages in Figs 1 and 3. In Fig 4 the method of Northrop gave a more concave curve.

It is therefore concluded (1) that permeability to water increased

² To be reported later

with age by a linear series of small increments, essentially equal during early and intermediate ages, progressively greater during late ages, (2) that the values given above are essentially correct. It is not improbable that permeability of all cells increases with age. It is extremely significant that increased water intake with age also occurs in inorganic and organic hydrophylic colloids (Dhar (24))

Later studies will discuss the consequences of this increasing permeability of aging eggs

CONCLUSIONS

1 Unfertilized *Arbacia* eggs, subjected to 50, 60, or 80 per cent sea water, at the same temperature, pH, and duration, swelled progressively faster with age. Swelling increased 12.2 to 39.6 per cent.

2 Permeability to water was calculated by the method of Lucké and McCutcheon. For freshly shed eggs from nine different females, permeability varied from 0.078 to 0.124, or 59 per cent. This variation was not due to differences in age nor to physiologic condition but was correlated with size of eggs.

3 About twenty eggs were tested each age to 60 hours after shedding. The permeability rate increased with age in all nine experiments.

4 The total increase ranged from 30 to 69 per cent.

5 The increase was slow but constant or almost constant during early and intermediate ages.

6 The increase began at earliest ages.

7 An increase may not be demonstrable for any short interval but is readily demonstrable for longer intervals (6 or more hours), or, from the entire curve.

8 Permeability increased faster at late ages. For special factors during late ages see text.

9 Injury by experimental procedure was not induced until *ca.* the 24th hour. Injury by aging was not detectable during the first 6 to 9 hours. It increased progressively between 10 and 24 hours, and very rapidly thereafter. Permeability increased at approximately the same rate during early and intermediate ages. Permeability therefore was not correlated with degree of injury.

10 The troublesome factor in the permeability equation was initial size, or V_0 . Several methods were used to obtain this V_0 , *viz.* (a)

extrapolations approximating known cyclical change in size, (b) extrapolations approximating control sizes, (c) constant or average of all V 's, (d) maximum V approximating control V 's. Recalculation with these different V 's gave approximately the same permeabilities.

11 The increase in permeability with age was substantially the same whether "approved," or other possible rates or average rates or range of rates be used as criteria of change.

12 When the data were recalculated by integrating the equation of Northrop, the increases in permeability with age were essentially the same as the increases calculated by the graphic method of Lucké.

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THE ACCUMULATION OF ELECTROLYTES

VIII THE ACCUMULATION OF KCl IN MODELS

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Potassium is usually said to accumulate when its concentration becomes greater inside the living cell than outside. But as the conception of accumulation usually appears to imply an expenditure of energy (and is chiefly interesting for this reason) it would seem more logical to confine it to those cases where the chemical potential of a potassium compound, *e g* KCl, inside the cell actually shows an excess over that outside. In that case we should speak of the accumulation of KCl but not of the accumulation of potassium since potassium might enter and reach a higher concentration inside, as in the Donnan equilibrium, although no potassium compound attained an excess of chemical potential inside.

Potassium chloride accumulates in many living cells. A favorable object for study is the marine alga *Valonia macrophysa*, Kütz, whose large multinucleate cells reach the size of a pigeon's egg and whose sap can be obtained with little or no contamination. The sap contains¹ approximately 0.5 M potassium or about 40 times as much as the sea water, but the sodium is only 0.1 M or about one fifth that of the sea water. The chloride is 0.6 M or about 3 per cent more than in the sea water (the latter is about 0.58 M²).

It is evident that the chemical potential of KCl is greater inside

¹There is also a little organic matter and possibly a trace of calcium. Cf. Osterhout, W J V, *Ergebn Physiol* 1933, 35, 981.

²Both the sap and the sea water are subject to fluctuations (Jacques A G, and Osterhout, W J V, *J Gen Physiol* 1930-31 14, 301, Osterhout W J V, *Biol Rev*, 1931 6, 370).

since (putting concentrations in place of activities³) we have for the product $(K)(Cl)$ inside $(0.5)(0.6) = 0.3$ and outside $(0.012)(0.58) = 0.007$

To make the chemical potential of KCl greater inside requires energy. The cell has energy at its disposal but the problem is to discover how it is applied in order to bring about this result.

One way of attacking this problem is to experiment with models in which KCl accumulates. In these, as previously described,⁴ two aqueous phases (an outer, A , and an inner, C) are separated by a non-aqueous phase, B , containing 70 per cent guaiacol plus 30 per cent p -cresol (this will be called $G-C$ mixture).

It is desirable to imitate the conditions in *Valonia* where the external solution contains much more chloride than potassium, the chloride being paired with a cation which enters much more slowly than potassium. In the case of *Valonia* this cation is sodium, but in the model lithium was used because then the relative rates of entrance of the cations become more nearly like that in *Valonia*⁵ although the difference between the two cations is much less than in *Valonia*⁶ and it is perhaps partly for this reason that we are unable to imitate its composition in the steady state more closely.

When we place KOH in A it unites with the organic acids in B (these will be called collectively HG) according to the equation $KOH + HG \rightleftharpoons KG + H_2O$ and KG then passes through B to C . We therefore, for convenience, placed KG instead of KOH in A which contained $0.05\text{ M } KG + 0.10\text{ M } LiCl$. This solution flowed slowly through A so as to keep its composition approximately constant.

These substances all passed through B into C , which contained only

³ This is permissible for comparative purposes since the ionic strength of the sap and the sea water are so nearly alike. Cf. Zscheile, E. P., Jr., *Protoplasma*, 1930, 11, 481.

⁴ Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931-32, 15, 667.

⁵ In *Valonia* the rate of entrance of potassium as compared with sodium is far greater than in the model (Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, 35, 991). The rate of entrance of lithium in the model is a little slower than that of sodium. Cf. Osterhout, W. J. V., Kamerling, S. E., and Stanley, W. M., *J. Gen. Physiol.*, 1933-34, 17, 469.

⁶ Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, 35, 991.

⁷ A , B , and C were stirred. For experimental details see Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931-32, 15, 667.

distilled water through which air was bubbling⁸ Evidently in the course of time *C* would become identical in composition with *A* To hasten this process the distilled water was removed from *C* and replaced by solution taken from *A*⁹ As in all the experiments described in this paper the solution in *A* was continually renewed and *A*, *B*, and *C* were stirred

We then bubbled CO_2 through *C* This reacted with *KG* to form KHCO_3 We may therefore for convenience regard the process as consisting of two reactions, (1) $\text{KOH} + \text{HG} \rightleftharpoons \text{KG} + \text{H}_2\text{O}$, and (2) $\text{KG} + \text{H}_2\text{CO}_3 \rightleftharpoons \text{KHCO}_3 + \text{HG}$ As HG in the system is constant this reduces to $\text{KOH} + \text{H}_2\text{CO}_3 \rightleftharpoons \text{KHCO}_3 + \text{H}_2\text{O}$ It is evident that potassium will continue to move from *A* to *C* as long as the chemical potential of KOH is greater in *A* than in *C* The chemical potential of KOH in *C* is kept lower than that in *A* by two factors, (1) CO_2 is constantly supplied in *C*, (2) when KHCO_3 is formed in *C* it lowers the activity of the water and raises the osmotic pressure and in consequence water moves from *A* to *C*

As a result we find that potassium moves from *A* to *C* throughout the entire experiment and the volume of *C* steadily increases This may be analogous to what happens in *Valonia* What is said regarding potassium applies also to lithium but the latter enters less rapidly because the partition coefficient (between *A* and *B*) of LiG is less than that of KG ,¹⁰ as would be expected, according to Shedlovsky and Uhlig,¹¹ on account of its smaller ionic radius

The course of events (Experiment 168) after the bubbling of CO_2 began is made evident by Figs 1, 2, and 3 the last shows that little or no chloride moved through *B* in either direction since the number of millimoles in *C* remained practically constant¹ But as water

⁸ Gases bubbled through *C* first passed through a solution of approximately the same composition so that they neither added nor subtracted water from *C*

⁹ *B* was brought into approximate equilibrium with *A* by being shaken with it and being allowed to stand overnight

¹⁰ Osterhout, W J V, Kamerling, S E, and Stanley, W M, *J Gen Physiol*, 1933-34, 17, 469

¹¹ Shedlovsky, T and Uhlig, H H, *J Gen Physiol*, 1933-34 17, 563

¹² Chemical determinations were made as described in a previous paper (cf footnotes 7 and 10) Lithium was determined by difference after determining HCO_3 by titrating with acid Cl was determined by potentiometric titration with AgNO_3 , glucose was determined by Benedict's method

moved from *A* to *C* (as shown by the volume curve in Fig 1) the concentration of chloride in *C* fell off

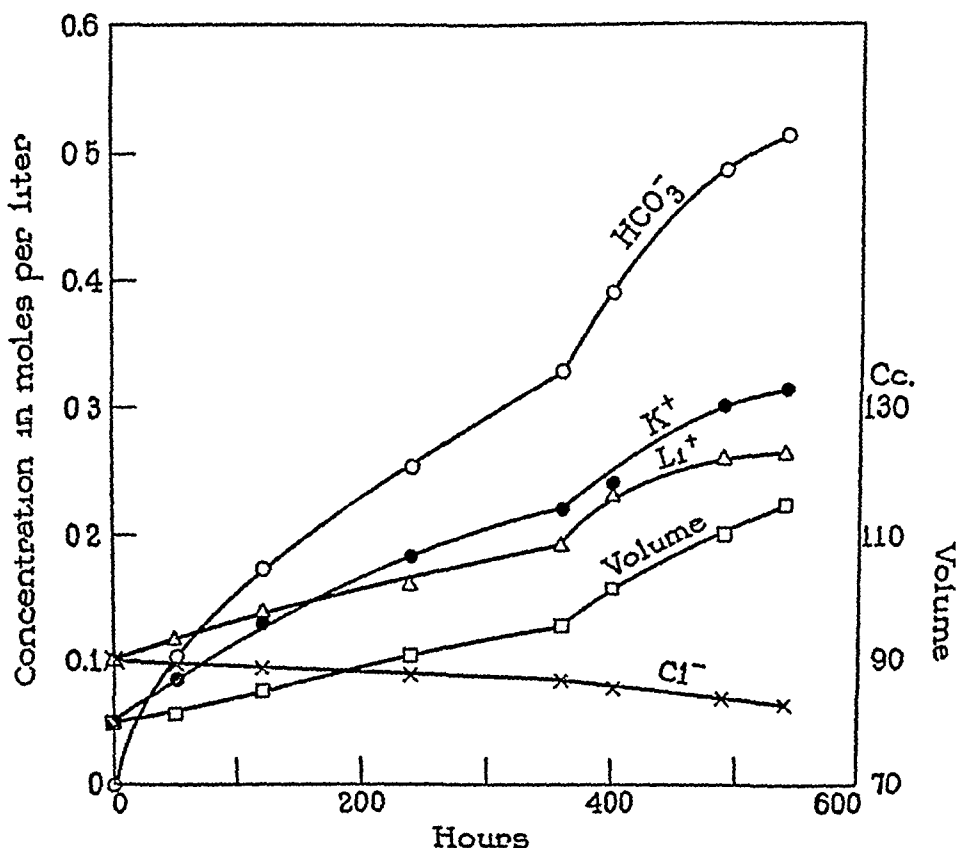


FIG 1 Shows changes in concentration (Experiment 168) in the inner aqueous phase *C* of a model consisting of an outer aqueous phase *A* which is separated from *C* by a non-aqueous phase *B* (consisting of 70 per cent guaracol + 30 per cent *p*-cresol these are called collectively HG) KG passes from *A* through *B* to *C* where it reacts with CO₂ to form KHCO₃ (this also applies to LiG), water also enters as shown by the volume curve At the start *A* and *C* have the same composition, *i.e.*, 0.1 M LiCl + 0.05 M KG At 240 hours carmine was added to *A* to determine whether any mechanical transfer occurred as none was found the carmine was removed at 360 hours and thereafter the rate of stirring was a little faster causing a more rapid increase in concentration

In order to see whether there was any mechanical transfer of liquid from *A* to *C* (due to the stirring) carmine was added to *A* at 240 hours As this is only slightly soluble in *B* its presence in *C* would indicate

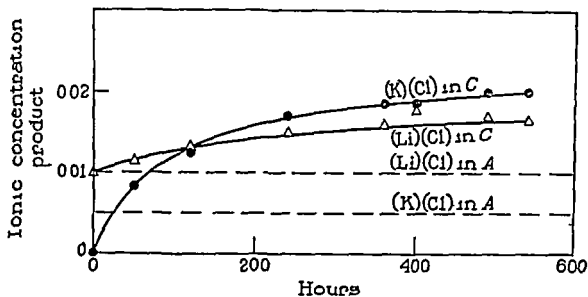


FIG 2 As in Fig 1, but showing the values of the ionic concentration products $(K)(Cl)$ and $(Li)(Cl)$ in A and in C. At the close of the experiment the value of $(K)(Cl)$ in C was about 4 times that in A.

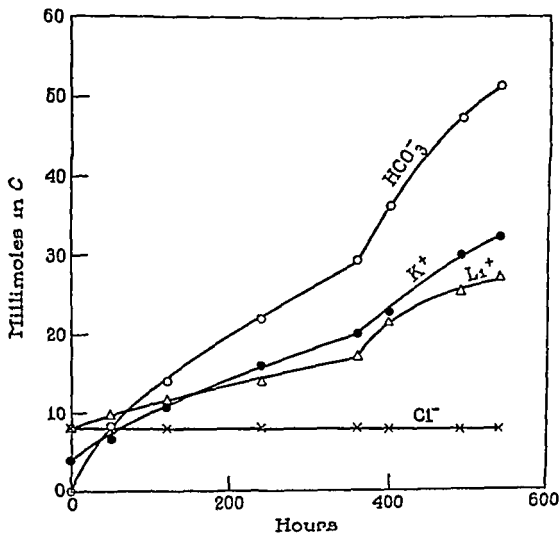


FIG 3 As in Fig 1, but showing millimoles in C.

the amount of mechanical transfer. It became evident that no such transfer occurred and it was consequently removed at 360 hours. In order to do this *A*, *B*, and *C* were separated and when put together again the rate of stirring was a little greater, thus causing the increase in rate shown in Fig. 1 after 360 hours.

Let us now consider the chemical potential, which is proportional to the ionic activity product $[K][Cl]$ ¹³. Fig. 2 shows the value of the ionic concentration product $(K)(Cl)$ outside and inside. At the close of the experiment (538.5 hours) the ionic concentration product was $(0.313)(0.0636) = 0.0199$ in *C* or about 4 times as great as the product in *A* which was $(0.05)(0.10) = 0.005$. We can get an approximate idea of the ionic activity products if we assume that the activity coefficients for K^+ and Cl^- are equal¹⁴ and are the same as in a solution of the same ionic strength with KCl as the only solute. In that case we should have for the activity coefficient of K^+ and Cl^- in *A* (with an ionic strength of 0.15) 0.735 and for *C* (with an ionic strength¹⁵ of 0.575) 0.634. On this basis the ionic activity product for *C* is $(0.0199)(0.634)^2 = 0.008$ or about 3 times that in *A* which is $(0.005)(0.735)^2 = 0.0027$.

Let us now consider lithium. At the close of the experiment the ionic concentration product $(Li)(Cl)$ was $(0.1)(0.1) = 0.01$ in *A* and $(0.262)(0.0636) = 0.0166$ in *C*¹⁶. As we are not justified in putting the activity coefficient of Li^+ equal to that of Cl^- there is no basis for calculating the ionic activity products.

During the experiment the volume increased from 80 to 113.5 cc. If this could have been prevented larger values of the product $(K)(Cl)$ in *A* might be expected. In order to test this we repeated the experiment, adding 0.5 M glucose to *A* (Experiment 169A). At the close of the experiment (505.5 hours) the volume had increased from 80 to 97 cc (a less increase than in the previous experiment). The ionic

¹³ Cf. footnote 4.

¹⁴ This assumption, originally made by D. A. MacInnes, is now frequently employed.

¹⁵ This is made up of $K^+ = 0.313$ M and $Li^+ = 0.262$ M or of $HCO_3^- = 0.512$ M and $Cl^- = 0.0636$ M.

¹⁶ The concentration of HCO_3^- was 0.512 M, the concentration of HG was about the same as in *A*, i.e., about 0.1 M.

concentration product $(K)(Cl)$ was $(0.313)(0.0782) = 0.0245$ in C and $(0.05)(0.1) = 0.005$ in A . The activity coefficient for K^+ and for Cl^- in A (with an ionic strength of 0.15) was taken as 0.735 and that in C (with an ionic strength¹⁷ of 0.58) as 0.632. Hence if we calculate the ionic activity products on the same basis as before we have for C $[K][Cl] = 0.0245(0.632)^2 = 0.0098$ and for A $(0.005)(0.735)^2 = 0.0027$. Here the calculated chemical potential in C is about 3.6 times as great as in A as compared with about 3 times in the previous experiment. Since the addition of glucose to A decreases the activity coefficients of K^+ and Cl^- the value 3.6 should be regarded as too small.

In this case the ionic concentration product $(Li)(Cl)$ was $(0.1)(0.1) = 0.01$ in A and $(0.267)(0.0782) = 0.021$ in C .

The addition of 0.5 M glucose¹⁸ to A did not greatly reduce the rate of entrance of water and it is evident that the addition of more would have produced more favorable results. This, however, was not possible because it would have too greatly increased the specific gravity of A which rests on a layer of $G-C$ mixture in the model.

As in the previous experiment, the millimoles of Cl^- in C remained practically constant but the concentration fell off from 0.1 M to 0.0782 M as the result of the entrance of water (less than in the previous experiment where it fell from 0.1 to 0.0636).

That the system is capable of raising the chemical potential of KCl to a higher level is evident from an experiment (No. 165) which started out with the same solution in A and in C , namely, 0.1 M $LiCl + 0.05$ M KG (before the start B was brought into approximate equilibrium with this solution by being shaken up with it and being allowed to stand overnight).

Air was first bubbled through C as this produced no change. CO_2 was bubbled through C with the result shown in Figs. 4, 5, and 6. Although the millimoles of Cl^- in C increased somewhat the entrance of water (as shown by the volume curve in Fig. 4) was so rapid that the concentration of Cl^- fell off. As long as the entrance of K^+ and

¹⁷ This is made up of 0.313 M K^+ and 0.267 M Li^+ or of 0.502 M HCO_3^- and 0.0782 M Cl^- .

¹⁸ At the end of the experiment C contained about 0.042 M glucose.

Li^+ was more rapid than that of water the ionic concentration products $(\text{K})(\text{Cl})$ and $(\text{Li})(\text{Cl})$ continued to increase (Fig 5), but as the rate of entrance of cations fell off sooner than that of water these products declined after a time. As would be expected this took place sooner with Li^+ than with K^+ since Li^+ entered more slowly

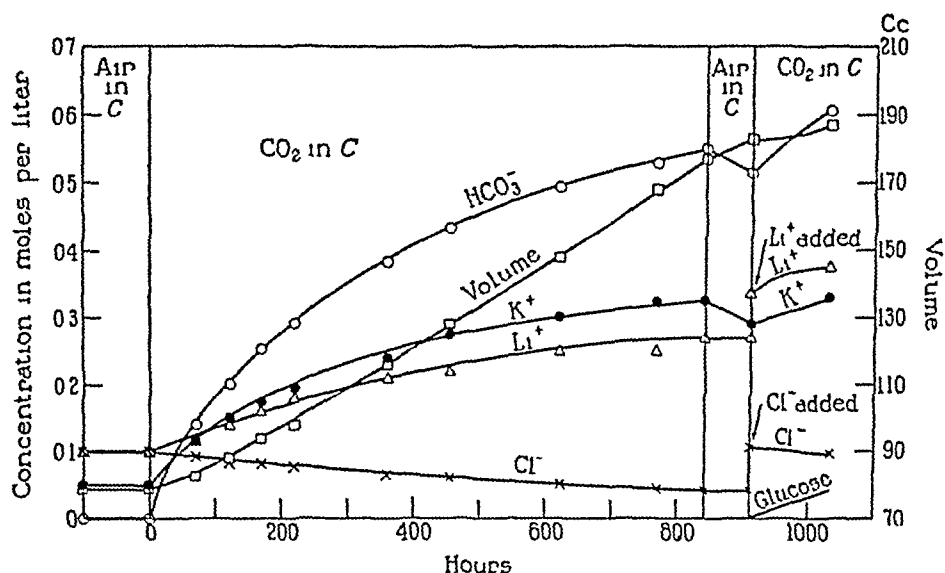


FIG 4 At the start the experiment was arranged as in Fig 1. At first air was bubbled in C and this produced no change. CO_2 was then bubbled through C and 840 hours later the bubbling of CO_2 in C was stopped and air was bubbled instead. This caused a falling off in the concentration of K^+ and of HCO_3^- . At 910 hours the bubbling of CO_2 was resumed, 0.5 M glucose was added to A and LiCl was added to C, increasing the Li^+ in C from 0.27 M to 0.337 M and Cl^- from 0.04 M to 0.107 M. After this K^+ continued to enter and at 1032 hours the ionic concentration product $(\text{K})(\text{Cl})$ in C was 0.033 or 6.6 times that in A (which was 0.005). This is shown in Fig 5 (Experiment 165).

At 840 hours we began to bubble air in C in place of CO_2 and there was a marked falling off in the concentration of K^+ and of HCO_3^- but the volume continued to increase as would be expected in view of the smaller activity of water in C.

At 910 hours enough glucose was added to A to make its concentration 0.5 M and LiCl was added to C, increasing the molality of Li^+ from 0.27 to 0.337 and that of Cl^- from 0.04 to 0.107. This raised the level

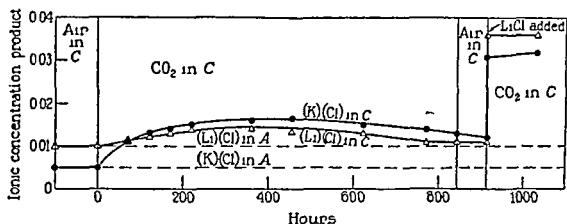


FIG 5 As in Fig 4 but showing the ionic concentration products $(K)(Cl)$ and $(Li)(Cl)$ in A and in C

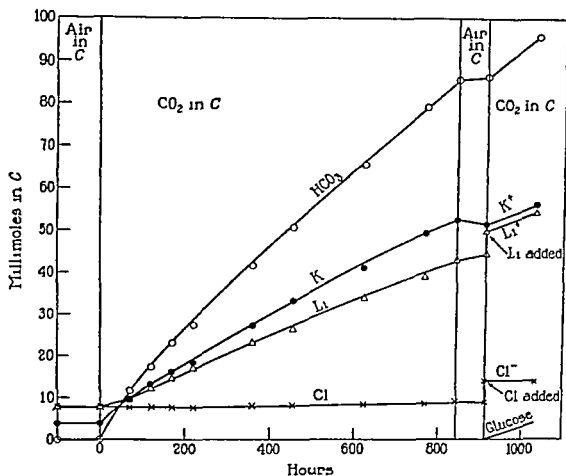


FIG 6 As in Fig 4 but showing millimoles. The millimoles of Cl^- increased somewhat after the start

of chemical potential of KCl in *C* but the system had sufficient energy to raise it still further, as shown by the increase in the product (K)(Cl), although water continued to enter (as shown by the volume curve in Fig. 4)

At 1032 hours the ionic concentration product (K)(Cl) was (0.33)(0.098) = 0.033 in *C* or 6.6 times as great as in *A*. If we calculate the ionic activity products as before we obtain 0.0125 for *C* or 4.64 times as much as for *A*. This latter is taken¹⁹ as 0.0027, but the value should be lower because the glucose lowers the activity coefficient; hence the value 4.64 should also be higher.

Evidently the system has sufficient energy to raise the chemical potential of KCl in *C* to a high level and this would occur in all the experiments if the entrance of water could be sufficiently restricted. This applies to experiments in which *C* consists at the start of distilled water through which CO₂ is bubbled (i.e. there is no K⁺, Li⁺, or Cl⁻ in *C* except what enters through *B*). Such experiments were performed and, even without adding glucose to *A*, we obtained an excess of chemical potential of KCl in *C*, but as the value of the ionic activity product in *C* in no case exceeded that in *A* by more than 20 per cent and as there is some uncertainty regarding the value of the activity coefficients²⁰ of K⁺ and Cl⁻ we prefer to lay no stress on these results. Higher values were obtained when the G-C mixture was allowed to stand in contact with dry powdered alfalfa leaves for some hours before starting the experiment, but it is a question whether the G-C mixture became a better carrier of Cl⁻ by this treatment or whether it merely gave up to *C* some of the Cl⁻ absorbed from the alfalfa.

¹⁹ In *A* with an ionic strength of 0.15 the activity coefficient of K⁺ and of Cl⁻ is taken as 0.735 and the ionic activity product is (0.05)(0.1)(0.735)² = 0.0027. *C* had an ionic strength of 0.71 (made up of K⁺ = 0.33 M and Li⁺ = 0.38 M or of Cl⁻ = 0.098 M and HCO₃ = 0.607 M) and the activity coefficient of KCl was taken as 0.618. We therefore have for the ionic activity product (0.33)(0.098)(0.618)² = 0.0125.

²⁰ Strictly speaking it is impossible to measure activity coefficients of individual ions but mean ionic activities may be measured and in the case of KCl the assumption may be made that the activity coefficients of the anion and of the cation are equal (see footnote 14).

DISCUSSION

Energy is needed to make the chemical potential of KCl greater in *C* than it is in *A*. This energy is derived from the chemical reactions in the system and from the continual supply of materials, particularly of CO_2 , which keeps the pH inside lower than it is outside. It is possible that similar considerations may apply to the living cell where the sap usually has a lower²¹ pH than the external solution owing to the presence of CO_2 and of other organic acids. It should be noted that the energy developed in the formation of CO_2 is not available in the model as it is in the living cell (since in the model we use CO_2 already formed).

The amount of accumulation is affected by the entrance of water. In the plant cell the rate of entrance of water may be greatly restricted by the cellulose wall and the accumulation may be correspondingly increased.

In the plant cell both K^+ and Cl^- reach higher concentrations than inside. In *Valonia* the difference between Cl^- outside and inside is small (0.6 as compared with 0.58) but in *Nitella* it is much higher.²² In the model the concentration of Cl^- inside in no case exceeded that outside and when CO_2 was bubbled it became less. Whether a model can be constructed in which both K^+ and Cl^- reach a higher concentration inside remains to be seen. To accomplish this the entrance of Cl^- must be more rapid than that of water.

In conclusion it may be desirable to call attention to the fact that the electrolytes pass through *B* chiefly in the form of molecules²³ or of undissociated neutral complexes which do not increase the conductivity.²⁴ In the case of HCl, for example, these can form at the interface by the collision of H^+ and Cl^- just as when HCl passes from an aqueous solution into air.

²¹ In *Valonia* the difference of pH is about as great as in the models. Cf. footnote 4.

²² Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, 35, 981.

²³ It is the practice of some authors to speak of molecules where there is sharing of electrons as in the case of HCl and of neutral complexes where this is absent as in the case of KCl.

²⁴ Cf. Shedlovsky, T., and Uhlig, H. H., *J. Gen. Physiol.*, 1933-34, 17, 549, 563.

When the activity product $(K)(Cl)$ is greater inside than outside potassium tends to go out as KCl but at the same time it tends to enter as KOH because the activity product $(K)(OH)$ is greater outside than inside ²⁵ The net result is entrance of potassium, presumably because the latter process is more rapid This seems to correspond to the situation in *Valonia* ²⁶

SUMMARY

Models are described in which KCl enters until its chemical potential becomes much greater inside than outside The energy needed to accomplish this comes from the chemical reactions occurring in the system and the continual supply of certain materials An important factor is the maintenance of a lower pH value inside by means of CO_2 This may be analogous to what happens in some living cells

The concentration of K^+ becomes higher inside, as happens in many living cells, but the concentration of Cl^- does not and in this respect the model differs from many living cells As in *Valonia*, potassium tends to go out as KCl when the ionic activity product $(K)(Cl)$ is greater inside but at the same time it tends to enter as KOH since the activity product $(K)(OH)$ is greater outside The net result is entrance of potassium presumably because the latter process is the more rapid

²⁵ *E g* in Experiment 168 the pH in *A* was about 9.1 and in *C* (at the end) was about 7.3

²⁶ Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, 35, 984

THE EFFECT OF X RAYS ON CHROMOSOMES IN DIFFERENT STAGES OF MEIOSIS

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PLATE 1

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The following investigation is concerned with the nature of the submicroscopic changes induced in the nucleus during meiosis and mitosis by the action of x rays. The results indicate that cells irradiated in the early prophase of the first meiotic division (synaptene pachytene) are markedly more susceptible to the effects of x rays than other stages in the ontogeny of the cell. They also give some indication of the nature of one of the biological variables that has been so troublesome in attempts to determine the relationship of induced genetic and cytological effects to dosage, and the dimensions of the volume of the hypothetical portion of the cell sensitive to the lethal action of x rays (Wyckoff, 1931-32, Glocker, 1932, Glocker, Langendorff, and Reuss, 1933).

One of the difficulties encountered in work on the biological action of x rays is the lack of uniformity of material, since populations of cells in various stages of development have been used. In *Gastrea* it is possible to eliminate this variable. Each bud has six anthers containing pollen mother cells in approximately the same stage. The inflorescence is a spike with a sequence of buds in different stages arranged in an ascending spiral along the stem. The chromosomes are large, few in number, and show their chromonematic structure clearly. In all of the species used there are four long chromosomes of the same size and three very short ones (Marshak, 1934).

Mohr (1919) found that nuclei of very young spermatocytes (*Delecticus verrucivorus*) were rendered pycnotic by treatments with radium and with low temperatures which did not affect other stages. Similar indications had been obtained by Regaud and his coworkers

(1906 and 1908) studying the effects of x-rays on the testes of mammals. Such pycnosis was observed in *Gasteria* pollen mother cells in the early prophase of meiosis when treated with doses of x-rays of 1,000 r or more. Whether this is a cumulative effect of the linkage changes observed at low dosages or is due to the action of x-rays upon another constituent of the nuclear or cytoplasmic systems remains to be determined. Stone (1933) has observed that chromosome abnormalities are evident in the first meiotic anaphase of pollen mother cells 24 hours after treatment with x-rays although the same dose produced little or no effect on other stages. In the investigations of this author and the earlier work of Strangeways and his coworkers (1923, 1925) it is observed that the mitoses from nuclei irradiated in the "resting stage" have chromosome abnormalities. These results are in accord with the findings of the present investigation if the resting stage and what is here considered the early mitotic prophase be identical. There is little morphological difference between these early prophase stages and the resting stage. I refer to a condition of the nucleus which is still reticulate but in which the chromonemata are beginning to be resolved. There is a large subjective element in such a distinction.

A consideration that seems to have been overlooked by previous investigators is the possibility that the immediate effects of x-radiation on chromosomes may not be at once apparent. For example, the inactivation of the chromatin-producing mechanism at a given locus will not become evident until the new chromonemata are developed. Similarly induced chromonematic interchanges will not be microscopically visible until disjunction occurs.

Technique

The distribution of division stages was determined by making smear preparations,¹ of the anthers of every bud on a young spike. The following procedure was used with the material irradiated. Beginning with the lower end of the spike, an anther from each bud was examined until one with the pollen mother cells in the first meiotic metaphase was found. This bud was left on the plant and all

¹ Fixed 15-30 minutes in Taylor's modification of Flemming's solution, rinsed in water, and placed in 50 per cent alcohol for 30 minutes to 1 hour. Stained in crystal violet.

the older ones removed. The remaining buds were numbered according to their position on the stem. At intervals along the stem a bud was removed and examined to determine the approximate location of buds with pollen mother cells in the various stages of the meiotic prophase and the premeiotic mitosis. The spike was then exposed to x rays. The remaining anthers from the bud which had shown the metaphase figures were smeared $\frac{1}{2}$, 1, and 2 hours after radiation. An anther from the bud immediately above was then examined. If anaphase or metaphase figures were found the bud was removed and smeared and the next bud examined until one with its pollen mother cells in the pachytene was found. This was usually two to four buds above the first one with anaphase I figures. The bud was again examined 24 hours later when most of the cells were in the first meiotic anaphase. In some cases this proved to be somewhat too early or too late (cells in metaphase or telophase). When the spike was approaching its maximum elongation, usually about 11-14 days after radiation, it required approximately 48 hours for cells in pachytene to reach the first anaphase.

In order to make enough determinations it was necessary to use more than one species of *Gasteria*. They were found to be similar in chromosome number and morphology in the configurations at anaphase, and in the intervals between different stages on the spike. The species used were *G. lingua*, *G. glabra*, *G. disticha*, and *G. sp.* In this work only the long chromosomes were followed.

The source of x rays was an air cooled Coolidge tube with a tungsten target. The primary current was kept at 110 volts, 10 milliamps. The secondary voltage was determined from the spectrum obtained with a spectrograph containing a rock salt crystal. The shortest wave length was found to be 0.15 \AA u corresponding to a peak secondary voltage of 82,400 volts. The range of wave lengths was from 0.15 to 0.40 \AA u with the maximum density near the 0.40 \AA u region. In the calculations the mean wave length is taken as 0.30 \AA u .

The dosage was varied by altering the time of exposure and the distance from the target. Dosage was measured with a Victoreen dosimeter. I am indebted to Dr. J. C. Hudson for the determinations of the characteristics of the radiation used.

Observations

The effects of x-rays as observed in the first meiotic anaphase may be put into three groups, namely, chromosome attachments, (Figs. 2, 4, 5, and 7), fragmentation (Figs. 2, 7, and 8), and achromatic spots (Fig. 6) (similar to "secondary constrictions") in the chromonema.

When the frequencies of chromosome abnormalities produced in buds of different ages are examined it is evident that there are pronounced maxima in buds which reached anaphase I at 1 and 5 days after radiation. These buds when irradiated were in pachytene.

TABLE I*

Frequency of Abnormalities Observed in Anaphase I shortly after Irradiation

Dose, in r	Time after radiation	Attached chromatids	Fragments	Achromatic spots	Total abnormalities	Total number chromatid pairs
	hrs	per cent	per cent	per cent	per cent	
264	0 5	0 6	0	0 02	0 8	3,368
264	1	0 9	0	0	0 9	1,200
79	1	0 94	0 07	0 02	1 03	2,891
15 8	1+	2 2	0	0	2 2	1,691
2 6	1+	1 7	0	0	1 7	2,068
528	1	0 3	0 06	0 06	0 4	2,520
528	1 5	25 0	24 1	0	49 1†	112
528	2	26 4	4 2	7 7	38 3	72
147	2	6 1	0 09	0 3	6 4	1,184
30	2	0 9	0	0 1	1 0	3,952

* In Tables I, II, and III and Text-figs 1 and 2 fragments have been counted as effects in one chromatid. In Text-figs 3 and 4 and calculations from them each fragment is counted as the result of an effect in a pair of chromatids.

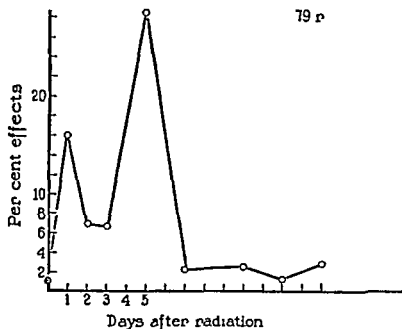
† At these dosages the frequency of fragmentation and spotting is so high that it is difficult to separate the two types of effects. For example an achromatic spot on a fragment may make it appear like two fragments. Obviously further investigation is necessary to determine whether there are definite maxima for achromatic spotting as for the other effects.

TABLE II

Frequency of Induced Abnormalities Observed in Anaphase I at Various Times after Irradiation with 79 Roentgens

Time after radiation	Total chromatid pairs	$\frac{\text{Attached chromatids}}{\text{Total}} \times 100$	$\frac{\text{Fragments}}{\text{Total}} \times 100$	$\frac{\text{Achromatic spots}}{\text{Total}} \times 100$	$\frac{\text{Total aberrant}}{\text{Total}} \times 100$
hrs					
1	2891	0 94	0 07	0 02	1 03
24	1776	15 88	0	0	15 88
48	1969	3 81	2 0	1 1	6 91
72	624	3 21	1 8	1 7	6 71
120	573	16 23	10 9	1 3	28 43
168	2022	0 89	1 2	0 2	2 29
240	1392	1 37	1 0	0 2	2 57
288	2648	0 80	0 4	0 06	1 26
336	928	1 72	1 0	0 1	2 82

stage of meiosis and in the early premeiotic prophase, respectively. In Table II and Text-fig 1 are shown the frequencies of abnormalities observed at the first meiotic anaphase in buds of the same spike removed at various times after treatment with 79 r and similarly for 528 r in Text-fig 2. Maxima at approximately the same time were also observed in buds exposed to 16, 30, and 264 r (Table III). In the 30 and 528 r series there are indications of peaks probably



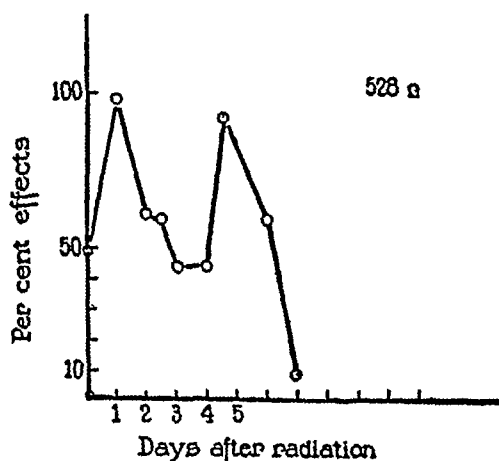
TEXT FIG 1 The frequency of chromosome abnormalities in x ray treated pollen mother cells (ordinate) plotted against time in days after irradiation (abscissa). The two peaks in the curves are taken to indicate periods of maximum sensitivity to γ rays. The first peak coincides with irradiation during the synaptene pachytene stage of meiosis the second with the early premeiotic prophase. Total dosage—79 roentgens.

corresponding to mitoses prior to the one shown by the buds which took 5 days to reach the first meiotic anaphase.

Of 6,723 pairs of chromatids from untreated plants of *G. lingua*, *G. conspurcata*, and *G. sp.* examined at the first meiotic anaphase, 0.5 per cent were attached so that the chromonematic coils were drawn out, 0.007 per cent (one case) had an achromatic spot in the chromonema, and there were 0.02 per cent of chromosome fragments. In Table I are given the frequencies of these chromosome aberrations observed shortly after irradiation. There is no marked increase

until somewhat more than an hour has elapsed between irradiation and examination of the cells

If the frequency of effects produced in buds rayed in pachytene be plotted against dosage, a straight line passing through the origin is obtained as the best fit to the points (Text-figs 3 and 4). A similar relationship is obtained from the values of the second peak (109 to 144 hours after radiation), given by buds irradiated during the premeiotic stage. Buds taken at 48 and at 72 hours after radiation also show a rectilinear relationship to dosage. Such a linear



TEXT-FIG 2 Data plotted as in Text-fig 1 Total dosage—528 roentgens

function is taken to indicate that photoelectrons produced in the absorption of x-rays within the tissue act directly² upon the biological agents involved in the binding together of the constituent portions of the chromonema. Although the linear relationship between dosage and the frequency of induced effects does not in itself prove a direct effect of x-rays on the biological units in question, any other explanation would involve assumptions which at present seem improbable.

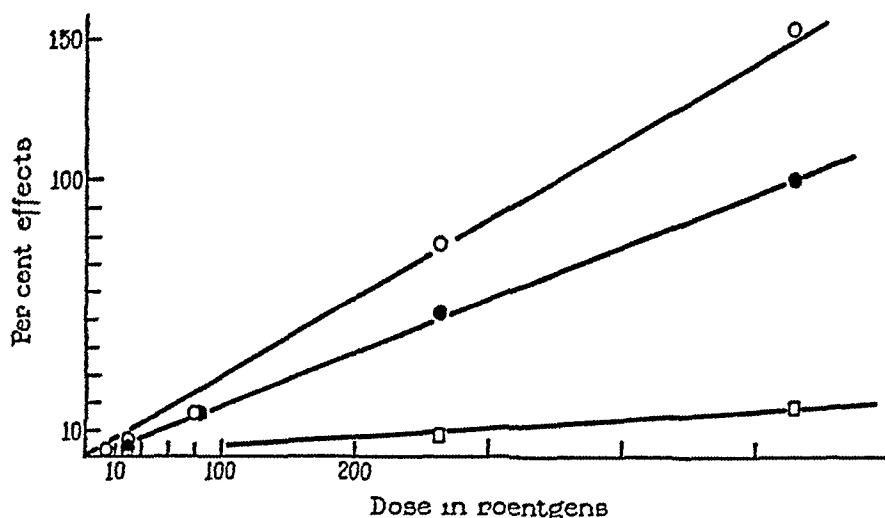
² We are not concerned here with the nature of the physical action of photoelectrons in the cells, *i.e.* whether effects are produced by ionization or other means, but with the problem of whether any particular category of effects observed is a consequence of the alteration of a single biological unit or system, or whether one system shows changes only after another has been modified by the radiation.

TABLE III*

Per Cent Total Chromosome Abnormalities with Different Dosages

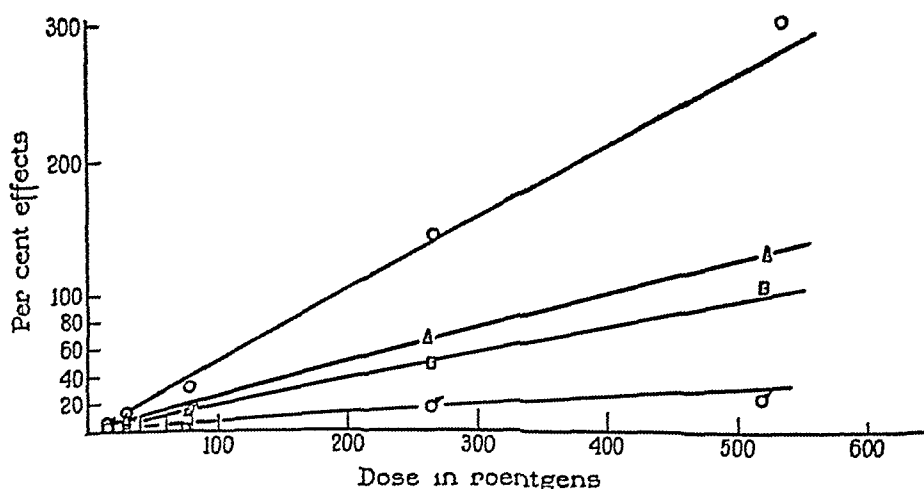
Time after radiation	Dose in r						
	2.6	5.3	15.8	30	79	264	528
hrs							
0.5						0.64	(0.46)
1	1.71				(1.1) 1.0	0.91	0.40 (73.2)
1.5							49.1
2				1.01		(76.9)	(152.6)
24			2.20		15.9	51.4	98.1
36				(6.5) 5.3 (3.4)			
48	0.97	1.35	0.98	3.0	(8.8) 6.9		(97.0) 61.2
60						(28.2) 18.5	(97.0) 59.2
72	0.74	0.26		(3.05) 2.75	(8.4) 6.7		(57.6) 57.0
96			(0.68) 0.60				(67.3) 44.1
109							(150.9) 92.6
120		(1.58) 1.24			(39.3) 28.4		
144			(1.65) 1.42	(4.1) 3.54			(97.8) 59.2
168				(3.93) 2.96 (1.36)	(3.4) 2.3		(13.6) 8.96
216				1.05 (0.71)	(3.5)		
240				0.52	2.6		
266							
288				(1.24) 0.80 (2.45)	(1.8) 1.3		
312				2.00			
336					(3.7) 2.8		
424				(0.81) 0.61			

* The figures in parentheses are the percentages of chromosome abnormalities when fragments are counted as resulting from effects induced in pairs of chromatids. Attached chromatids have in all cases been counted as effects in pairs of chromatids and achromatic spots as effects in single chromatids.



TEXT-FIG 3 The frequency of x-ray-induced chromosome abnormalities observed in anaphase I 24 hours after irradiation. These data represent effects induced at the synaptene-pachytene stage of meiosis.

Abscissae, dose in roentgens, ordinates, per cent effects. Open circles represent total chromosome abnormalities when fragments are considered as being produced by an x-ray effect on a pair of chromatids, closed circles the same totals when a fragment is considered as being produced from a single chromatid. Squares represent frequencies of achromatic spots. The first meiotic division is considered reductional.



TEXT-FIG 4 Circles represent total chromosome abnormalities as in Text-fig 3, but with the first division considered both equational and reductional with equal frequency. Circles with arrows represent chromosome attachments, rectangles fragments, and triangles attachments plus fragments, all with the first division always reductional.

There are no very pronounced maxima in the frequencies of induced achromatic spots at low dosages, although there is considerable variability probably due to the difficulty of detection at the higher dosages. There is a noticeably lower frequency in buds which are quite young when irradiated. The data for attachments and fragments are similar to those for achromatic spots in this respect and suggest that cells affected in any of these ways have a greater tendency to die off than non affected cells. In Text fig 3 the frequency of achromatic spots in buds examined 24 hours after irradiation is plotted against dosage. Here too the curve is rectilinear.

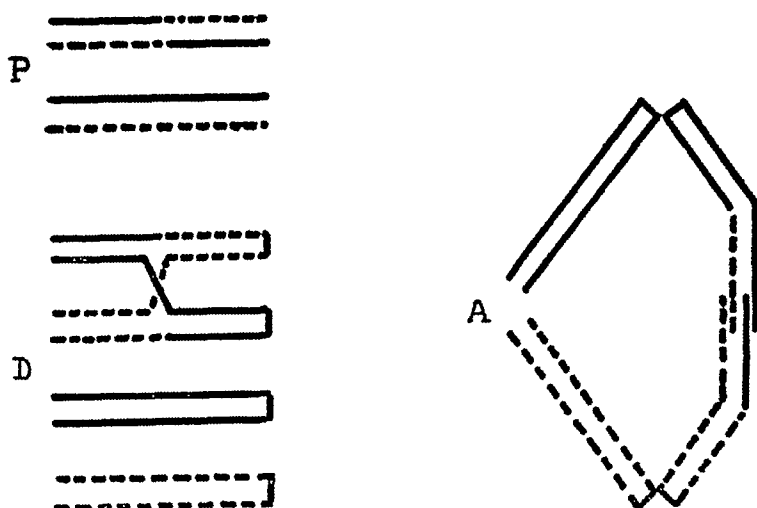
Interpretation

Accepting the hypothesis of direct action, it becomes necessary to explain by means of it the higher frequency of chromosome aberrations induced in cells irradiated at particular stages of division. Such an explanation must apply to some premeiotic condition as well as to the synaptene pachytene stage of the meiotic prophase. It must also account for the fact that there are no pronounced maxima in the frequency of induced achromatic spots.³ Of several schemes devised to indicate the manner in which the condition leading to attached chromonemata might be produced, only one seems to satisfy the conditions adequately. This requires that each synapsed homologue be at least two parted at the time of irradiation, that a change of linkage take place between a pair of strands when a photoelectron intersects both, and that a chromonematic division occur between this stage and anaphase, as illustrated in Text fig 5.⁴ Careful examination of anaphase chromosomes from unirradiated plants showed that the chromonema of each chromatid was visibly two parted at least along some portions of its axis (Fig 3). From the same untreated material there are visible indications that the attachment of chromonemata that leads to attenuation of the chromonematic helix is

³ There are indications of maxima for induced achromatic spots in the data from the 264 and 528 r series but not at the lower dosages. (See footnote to Table VI.)

⁴ It has been suggested that the chromatid attachments may be produced by reverse cross-overs, i.e., lateral attachments between chromonemata. This would require that every attachment be accompanied by a fragment. The data do not show such a correlation between fragments and attachments.

due to an interlocking of half-chromonemata (Fig 3) The condition in the x-rayed material (Fig 4) is very much the same, and it seems reasonable to suppose that the mechanisms immediately responsible are similar in the two cases In the irradiated cells there is another type of attenuation in which a portion of the chromonema becomes drawn out to a slender thread which finally breaks, leaving the distal portion of the chromatid pair on the median plate (Figs 5, 7, and 8) Since chromonemata may remain attached through the second meiotic anaphase (as far as they were followed), it seems possible that



TEXT-FIG 5 Diagram to illustrate formation of attached chromatids Dotted and solid lines represent homologous chromatids *P* = pachytene, *D* = diplotene, *A* = anaphase

we are dealing with two really different conditions The type of attenuation leading to fragmentation may be produced by assuming an inactivation of the chromonematic division mechanism in the region of a pair of chromonemata intersected by a photoelectron, which may or may not be followed by another division in the rest of the chromonema Another explanation alternative to the inactivation of division might be the alteration of forces holding the chromonema together, *i e*, weakening of linkage In either case pairs of chromonemata must be affected, since the condition observed is usually an attenuation of a portion of both members of a pair of chromatids The schemes for both types of attenuation require the presence of

pairs of very intimately associated strands, a condition which may be common to the early prophase of the last premeiotic mitosis and the pachytene stage of meiosis. In the one case (mitosis) the close association may be considered to be due to the division of the units of the chromonematic strand forming the new units of the still microscopically undetectable chromonema. In meiosis the condition is produced by synapsis or by another chromonematic division.

Since attenuations are in pairs of chromatids,⁵ not threes or fours, there follows as a result of this interpretation an interesting implication concerning the nature of synapsis, namely, that although there is a pairing of four strands, at any given locus there is a very intimate association between only two of them. It appears as though the association between the members of a pair of strands is of a different order of magnitude from the association of the two pairs of the tetrad, although this difference cannot be detected optically in the pachytene chromosome. If the members of the intimately associated pair are different at different loci, it follows that there will be an alternate close association between homologous and sister chromatids. If under particular conditions the members of a pair are the same for a considerable length of the chromosome the more or less secondary association of the pairs might not, along this region of the chromosome, be an exact locus for locus combination. The "non homologous" pairing described in *Zea* (McClintock, 1932) may be interpreted as such a condition.

The achromatic spots observed in the x rayed chromonemata may be considered as originating from an inactivation of the chromatin producing mechanism in localized regions of these chromonemata. If each chromonema is single at or prior to pachytene, one would expect the achromatic spots to occur in pairs in the 24 hour buds. This does not agree with the observations. On the other hand, if only one of the four pachytene strands is affected at a given locus one might expect by the previous schemes that the newly formed chro-

⁵ At the lower dosages attachments occur only between pairs of chromatids in the 24 hour buds, but in the 48 hour buds attachments in threes do occasionally occur. For example, there were two complexes of three chromatids to twenty seven of pairs of attached chromatids at 48 hours 15.8 r, and thirty-eight pairs of attached chromatids and no complexes of three at 24 hours.

monema would replace the necessary constituent of the system altered by the photoelectron. However, it is possible that the chromatin-producing system may be functionally related to the reproductive one of the chromonema in such a way that when one is altered the other is also affected, or, that the production of chromatin or its precursors requires the presence of both chromonematic units at a given locus.

Sensitive Volume of the Chromonema

An estimate of the sensitive volume of the chromonema was obtained by using the equation given by Glocker (1932) for the number of photoelectron paths traversing a given volume

$$(1) N = N_0 \frac{R + a}{a} v,$$

where

v = the volume in question

N = number of electron paths in v

N_0 = photoelectrons per cm^3 tissue per roentgen

R = range of photoelectron in tissue

a = mean path length of photoelectron inside volume $v = \frac{4}{3} r$

The energy per roentgen per cm^3 air at 0°C , 760 mm Hg is taken as 0.11 ergs (Kuhlenkamp, 1929), and the density of the nucleus as approximately 1.035 (Harvey, 1932), of air as 0.001293. To get the relative electron density of tissue as compared with air the atomic composition of the cells used is taken as approximately that of a young embryo (Armsby and Moulton, 1925) and an average value for b of the equation $V_o' - V_x' = bx$, where $b = \frac{\text{Density} \times \text{atomic number}}{\text{Atomic weight}}$

is obtained as follows

Atom	Density	Atomic No	Atomic weight	b
	<i>per cent</i>			
O	70.3	8	16	39.6
H	10.3	1	1	10.3
C	5.1	6	12	2.55
N	1.3	7	14	0.65
Ash	2.2*	17*	16*	2.34
				<hr/> 55.44

* Values are the weighted means of the constituents of the ash

b for *Arbacia* eggs is somewhat less (though it can only be estimated from the data given by Harvey), while b for air is 60.41. Then taking $\lambda = 0.3 \text{ \AA u}$, $E = 6.54 \times 10^{-8}$ ergs, and $N_e = \frac{0.11}{6.54 \times 10^{-8}} \times \frac{1.035}{0.00129} \times \frac{55.4}{60.4} = 12.4 \times 10^8$

Using Glocker's method in which a correction is made for the argon content of air but the cell is considered as water at a density of 1.0,

$$N_e = 3.82 \times 10^9 \lambda \text{ in \AA u} \\ = 11.5 \times 10^8$$

R for air is calculated from the equation

$$R = \frac{V_o^4}{a'} \text{ where } V_o = \text{velocity of photoelectron}$$

and $a' =$ number of electrons per unit volume of material traversed $= 5.5 \times 10^{29}$ (Compton, 1926, Wilson, 1923)

$$\text{In air } R = \frac{2.1 \times 10^{40}}{5.5 \times 10^{29}} = 3.78 \text{ cm}$$

$$\text{in tissue } R = 3.78 \times \frac{0.00129}{1.035} = 4.7 \times 10^{-4} \text{ cm}$$

Glocker's relationship, obtained from Kühlenkamp's determinations $V = 21.4\sqrt{R(\text{air})}$, gives $R = 46 \times 10^{-4} \text{ cm}$

Using $N_e = 12.4 \times 10^8$, $R = 46 \times 10^{-4} \text{ cm}$, and $N = 2.8 \times 10^{-3}$ obtained from the slope of the uppermost line in Text fig 3, equation (1) may be solved by considering the volume spherical and solving for r with the result that $r = 1.25 \times 10^{-5} \text{ cm}$. The total sensitive volume of the pachytene chromosome (one of the pair of homologues) is $v = 8.2 \times 10^{-16} \text{ cm}^3$ if the first anaphase is reductional. If chromatids disjoin at random, $N = 5.2 \times 10^{-3}$ (derived from Text fig 4, uppermost line) and $r = 1.7 \times 10^{-5} \text{ cm}$, and $v = 20.6 \times 10^{-16} \text{ cm}^3$

As measured under the microscope the diameter of the pachytene chromosome is $13 \times 10^{-6} \text{ cm}$. The chromosomes at this stage are too long to be measured directly but an estimate of their approximate length can be obtained if analogies are made with the behavior of other meiotic chromosomes. The small chromosomes in *Gasteria* are essentially small sections of the long ones. They are also very similar in appearance to those of *Zea mays*. The length at pachytene could be measured from photographs of the B chromosome of *Zea*

which doubled back upon itself (McClintock, 1933, Fig 2) This was compared with the length of the metaphase chromonema, assuming that the relative length of the chromonema to the external dimension of the chromosome was the same as in *Gasteria* Making these assumptions one may determine the reduction in length of the chromonema from pachytene to metaphase, which proves to be a reduction of 11 times Knowing the length of the *Gasteria* metaphase chromonema by measurement, the length at pachytene may then be estimated as 21.2×10^{-3} cm

The meiotic nucleus in *Gasteria* is spherical and can be measured accurately It has a diameter of 35×10^{-4} cm and a surface area therefore of $a'' = 3.85 \times 10^{-5}$ cm² The number N' of photoelectrons traversing the nuclear volume may be calculated from equation (1) This may be taken as the number of photoelectrons intersecting the surface of the nucleus Assuming a cubical cross-section for the pachytene chromosome, it will have an area on one face of $a''' = 27.6 \times 10^{-8}$ cm² Since $N' = 82.5$, the number of photoelectrons entering the pachytene chromosome will be approximately 5.9×10^{-1} per roentgen

If w be the width of the sensitive volume v of a pair of chromonemata whose volume is v' , then

$$v = v' \times \frac{N}{N'}$$

$$l \times w \times \frac{w}{2} = l \times 1.3 \times \frac{1.3}{2} \times 10^{-10} \times \frac{2.8 \times 10^{-3}}{5.9 \times 10^{-1}}$$

$$\text{and } w = \sqrt{\frac{1.3 \times 1.3 \times 10^{-10} \times 2.8 \times 10^{-3}}{5.9 \times 10^{-1}}} = 9.0 \times 10^{-7} \text{ cm}$$

With random disjunction of chromatids

$$\begin{aligned} w &= \sqrt{\frac{1.3 \times 1.3 \times 10^{-10} \times 5.2 \times 10^{-3}}{5.9 \times 10^{-1}}} \\ &= 12.2 \times 10^{-7} \text{ cm} \end{aligned}$$

If the sensitive volume obtained by the previous method be dis-

tributed along the length of the chromonema the width of the chromonematic pair w' will be

$w' = \sqrt[3]{\frac{2v}{l}}$ where v = the sensitive volume of the pair of chromonemata and l their length

$$w' = \sqrt[3]{\frac{2 \times 7.22 \times 10^{-15}}{21.2 \times 10^{-3}}} = 8.8 \times 10^{-7} \text{ cm}$$

and with random disjunction

$$w' = \sqrt[3]{\frac{2 \times 20.6 \times 10^{-15}}{21.2 \times 10^{-3}}} = 13.9 \times 10^{-7} \text{ cm}$$

Although these values are obviously rough approximations it is interesting to compare them with the diameters of protein molecules and enzymes. Albumin = 4.34×10^{-7} cm (Svedberg and Sjogren, 1929, Nichols, 1930), trypsin = 5.2×10^{-7} cm (Northrop and Kunitz, 1932-33), insulin = 4.36×10^{-7} cm (Sjogren and Svedberg, 1931), hemocyanin = 2.4×10^{-6} cm (Svedberg and Chirnoaga, 1928)

The width of the sensitive portion of the single chromonema $\left(\frac{w}{2}\right)$ determined by either of the above methods falls well within the limits of the sizes of protein molecules and approximates the size of the smaller protein molecules and of insulin and trypsin. If we assume that the sensitive portion of the chromonema is composed of more or less spherical units we obtain for the number of such units $n = \frac{l}{\frac{w}{2}}$

which is 4.7×10^4 , 3.5×10^4 , 5.1×10^4 , and 3.0×10^4 for the different values of w . If the metaphase chromonema be considered to represent a condition in which there is a maximum condensation of sensitive material along the axis of the chromonema without any corresponding increase in width, then since the length of the chromonema is 1.93×10^{-3} cm, $n = 4.8 \times 10^3$, 3.2×10^3 , 4.7×10^3 , 2.8×10^3 . These two sets of values of n might be taken as representing the maximum and minimum limits of the number of such units in the chromonema, but since the shape and changes in size (if any) along different axes of such units are unknown the values have little meaning.

TABLE IV
Sensitive Volumes—Method II

Category	$N (\times 10^{-2})$	$w (\times 10^{-7} \text{ cm})$	$v (\times 10^{-1} \text{ cm}^3)$
Achromatic spots	0.3	2.9	0.9
Attachments	0.7	4.5	2.2
Fragments	1.9	7.4	5.8
Attachments and fragments	2.5	8.5	7.7
Total	2.8	9.0*	8.6

* Gowen and Gay (1933) have calculated the maximum size of a gene as $1 \times 10^{-13} \text{ cm}^3$, i.e., $w = 1 \times 10^{-6} \text{ cm}$. They considered the absorbed quantum as the agent effective in producing gene changes. If their calculations were made in terms of effects produced per ion pair the size would be smaller by a factor of about 500.

TABLE V
Per Cent Chromosome Fragments—Each Fragment Counted as Effect in a Pair of Chromatids

Time after radiation <i>hrs</i>	Dose in r						
	2.6	5.3	15.8	30	79	264	528
0.5						0	
1.0	0				0.1	0	0.1
1.5							48.2
2.0				0.03			
24			0		0	51.1	109.0
36				2.4			
48	0	0	0	0.8	3.9		71.7
60						19.5	75.3
72	0			0.7	3.5		29.2
96		0.03	0.09				46.2
109							117.0
120		0.3			21.8		
144			0.2	1.1			77.0
168				1.9	2.3		9.3
216				0.6			
240				0.4	1.9		
266							
288				0.9	0.9		
312				0.9			
336					1.9		
424				0.3			

In Table IV are given the results of calculations of sensitive volumes for the different types of observed effects whose frequencies are given in Tables V and VI. Fragmentation and attachments make up a large proportion of the abnormalities. If both these categories be

TABLE VI
Per Cent Achromatic Spots

Time after radiation <i>hrs</i>	Dose in r						
	2.6	5.3	15.8	30	79	264	528
0.5						0.02	
1.0	0				0.02	0	0.06
1.5							0
2.0				0.09			7.7
24			0		0	8.5*	18.3*
36				1.2			
48	0	0	0	0.8	1.1		10.1
60						2.8	9.4
72	0			1.0	1.7		13.3
96		0	0				10.6
109							22.4*
120		0			1.3		
144			0	1.0			4.2
168				0.6	0.2		1.3
216				0.2			
240				0.07	0.2		
266							
288				0.08	0.06		
312				0			
336					0.1		
424				0.05			

* Whether these apparent peaks are real or the result of confusion with fragmentation which is pronounced at these periods has not yet been proven. Since at the lower doses (30 r, 79 r) where such inaccuracy is reduced there are no pronounced maxima, it would seem that the apparent peaks are produced by the inclusion of fragments in this category.

considered consequences of effects in the linkage mechanism, then this system makes up about 90 to 95 per cent of the total sensitive volume of the chromonema. If the linkage mechanism is distributed primarily along the long axis of the chromonema, then its reduction

to a minimum will reduce the length of the chromonema to about one-tenth of its extended size. This is in good agreement with the reduction in length from pachytene to metaphase of 11 times as previously calculated. It would seem therefore that the contraction of the chromonema from prophase to metaphase is due largely to a reduction in the linear extension of the linkage material.

Of particular interest is the probable relation of these sensitive volumes to the genetic unit, whether this be a discrete body or a portion of one of the systems mentioned. If it can be shown that the frequency of x-ray-induced mutations varies with the distance between chromonemata then it would seem likely that the gene was a part of the linkage mechanism and that mutations were not changes in a more or less independent unit. For example one would expect a greater frequency of mutations in cells irradiated at pachytene or early mitotic prophase. There already exist indications of such a variation in mutation frequency from studies of effects on sperm and eggs in different developmental stages of *Drosophila melanogaster* (Moore, 1934, Hanson and Heys, 1929, Harris, 1929). If the genetic unit be considered a portion of the linkage mechanism one would expect unequal crossing-over and translocations to be accompanied by position effects of varying intensities, but if the gene is a discrete unit a position effect is not a necessary concomitant of unequal crossing-over or translocation.

SUMMARY

- 1 Pollen mother cells exposed to low dosages of x-rays at various stages show different frequencies of chromosome abnormalities in the first meiotic anaphase.

- 2 Maximum frequencies of abnormalities were obtained in buds irradiated in the pachytene stage of the meiotic prophase and in the preceding mitosis.

- 3 These results are taken to indicate that the x-ray-sensitive portions of the chromonemata are closely approximated in pairs in pachytene and in the early mitotic prophase. The significance of this in relation to non-homologous pairing is indicated.

- 4 From the nature of the chromosome configurations observed it is concluded that chromonemata are two-parted when they synapse.

and that a chromonematic division occurs between pachytene and anaphase and during the mitotic prophase

5 The frequencies of abnormalities show a linear relationship to dosage

6 The diameter of the sensitive volume of the chromonema is calculated and found to approximate the diameter of some known protein molecules

7 The linkage mechanism is found to make up about 90 per cent of the total sensitive volume which corresponds with the approximate reduction in length of the chromonema from pachytene to anaphase

8 The relation of these sensitive volumes to the gene is discussed

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EXPLANATION OF PLATE 1

Photomicrographs of meiotic chromosomes in *Gasteria*

FIG 1 Meiotic anaphase and early telophase—not irradiated.

FIG 2 Meiotic anaphase, telophase, and interphase—264 r—24 hours after irradiation

FIG 3 A chromosome with both pairs of chromatids attached to each other—not irradiated. Notice interlocking of half-chromatids

FIG 4 A pair of attached chromatids—79 r

FIG 5 A pair of attenuating chromatids—79 r

FIG 6 Same as (5) at another focus Arrow indicates an achromatic spot

FIG 7 Attenuating chromatids just broken (left) and about to break (right)—528 r

FIG 8 A fragment left on the equatorial plate—30 r



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THE DENATURATION OF EGG ALBUMIN BY ULTRA-VIOLET RADIATION

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It is a well known fact that exposure to ultraviolet radiation will denature a protein solution. Egg albumin, after such an exposure, shows a loss of solubility in water and will precipitate at the isoelectric point or, on half saturation with ammonium sulfate, at a pH somewhat removed from the isoelectric point (1, 2). Previous work on light denaturation has been largely of a qualitative nature and no satisfactory attempt has been made to separate the two steps involved in the process although Bovie (3) stated in 1913 that there are two steps in the coagulation of proteins by light, the first, the denaturation of the protein molecule, and the second, the flocculation of the denatured protein as a visible coagulum. At a pH removed from the isoelectric point only the first step takes place and the degree of denaturation can only be determined by precipitating the denatured molecules with ammonium sulfate or by bringing the radiated solution to the isoelectric point. If solutions are radiated at the isoelectric point flocculation follows denaturation more or less rapidly depending on the temperature of the solution.

This investigation was planned as a study of the temperature coefficient of the first step of the reaction and it was found that, by controlling the temperature at which the denaturation and subsequent flocculation were carried out, a quantitative analysis of the steps involved in the process could be made.

Method

The intensity of the Tyndall beam from the opalescent solutions was used to measure their degree of aggregation. This method has been frequently used (4, 5) and gives accurate results if the conditions are controlled. For very small par-

icles the strength of the Tyndall beam $= T = kcd^3$ and for large particles $T = k'c/d$, where c is the concentration and d is the diameter of the particles, so that the strength of the Tyndall beam is only proportional to the concentration if the size of the particles remains constant. Mecklenburg found that in a sulfur suspension of constant concentration the intensity of the Tyndall beam increased with the diameter of the particles from $d = 0.05 \times 10^{-5}$ cm to $d = 0.9 \times 10^{-5}$ cm but did not change appreciably between $d = 0.9 \times 10^{-5}$ cm and $d = 2.5 \times 10^{-5}$ cm. Tolman, working with a silica suspension of larger particle size, found that the intensity of the beam decreased with increasing diameter for particles larger than $d = 10 \times 10^{-5}$ cm. It would seem, therefore, that the region between 10^{-5} and 10^{-4} cm diameter is the region in which the law of scattering changes from that characteristic of small particles to that characteristic of large particles and that for this region the intensity of the Tyndall beam is practically independent of particle size. Emulsions have a diameter of the order of 10^{-4} to 10^{-5} cm and it has been found (6) that 4×10^{-5} cm is a

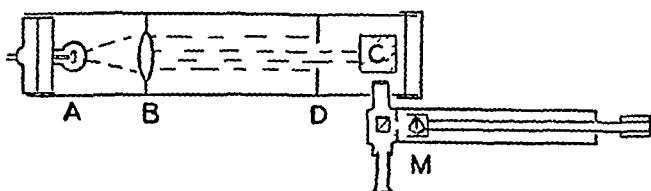


FIG 1 Diagram of Tyndallmeter. A = lamp, B = lens, D = diaphragm, C = cell (2 cm square), M = Macbeth illuminometer

critical size for equilibrium in oil emulsions, whether the emulsion is obtained by breaking down larger masses or by the coagulation of smaller particles. When Tyndall beam readings are made on an albumin solution during the process of flocculation the intensity of the beam increases at first, owing to increasing size of the particles, but finally reaches a constant value. When this condition is reached the particles begin to settle out but, if redispersed by shaking, the same constant value for the Tyndall beam is obtained. It would seem therefore that this constant value represents the reading obtained when the particles reach a critical size (10^{-4} to 10^{-5} cm diameter) and that in this region the intensity of the Tyndall beam is independent of the size and proportional to the concentration of aggregated protein.

Apparatus—An adaptation of the Tyndallmeter of Tolman and Vliet (4) was used and is shown in Fig 1. The meter consists of a 10 watt 120 volt Mazda bulb inserted at A in a metal tube and a 10 diopter lens placed at B, 10 cm from A, to give a beam of parallel light which passes through a diaphragm D and falls on the solution in the cell at C. The cell is a rectangular absorption cell 2 cm square (outside dimension). A Macbeth illuminometer is inserted at a hole opposite the side of this absorption cell to read the intensity of the Tyndall beam in

apparent foot-candles This instrument reads, with great accuracy, illuminations as low as 0.02 foot candles Solutions of undenatured, isoelectric egg albumin had a Tyndall beam reading of 0.03 to 0.04 apparent foot-candles Solutions were exposed in small quartz test tubes 1 cm diameter, placed inside and against the side of a quartz jar packed with ice and water, or filled with water at 14°C The source of radiation was a quartz mercury Uviarc at a distance of 6 inches

Crystalline egg albumin was prepared as described in a previous publication (1) After four recrystallizations with ammonium sulfate the crystals were dissolved in distilled water and dialyzed 2 days to remove the ammonium sulfate This gave a solution of undenatured isoelectric egg albumin of pH 4.8 To determine

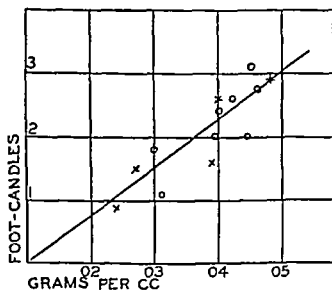


FIG 2

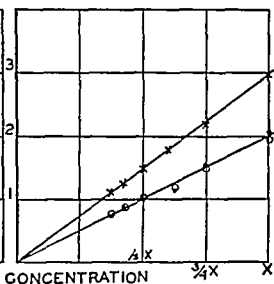


FIG 3

FIG 2 Standardization of Tyndall beam Ordinates, Tyndall beam in apparent foot candles Abscissae, grams of aggregated protein per cc (λ = results with Solution 1, \circ with Solution 2)

FIG 3 Standardization of Tyndall beam Ordinates Tyndall beam in apparent foot-candles Abscissae, dilutions of original concentration of λ grams per cc of aggregated protein (\circ and λ = results with two samples of Solution 1 and λ with Solution 2)

the concentration of the solution 10 cc. were boiled and the coagulum filtered and weighed The whole series of experiments was carried out with two separately prepared solutions the first a 1.1 per cent and the second a 1.01 per cent solution The results of the two series were in complete agreement

Standardization of Tyndall Beam—Two methods were used to prove that the intensity of the Tyndall beam is proportional to the concentration of the aggregated material after critical particle size is reached, in solutions where part of the albumin had been denatured and flocculated First, solutions of known Tyndall beam reading were filtered on weighed filter papers and the grams of aggregated material determined by drying to constant weight To check the results the

filtrates were boiled and the coagulum filtered and weighed to see that the combined weight of albumin in the precipitate and filtrate checked with the known concentration of the original solution. The results are given in Fig 2 and show that for both solutions used the Tyndall beam reading was proportional to the concentration, after critical particle size was reached, up to a beam of 3 apparent foot-candles which was equivalent to a concentration of 0.05 gm per cc. Above this point the solutions become so opaque that they absorb an appreciable fraction of the incident light and the Tyndall beam readings are no longer proportional to the concentration of aggregated protein.

The second method by which the intensity of the beam was shown to be equal to the concentration was by taking a solution of concentration X gm per cc and measuring the intensity of the Tyndall beam with progressive dilution. The results of three such experiments are given in Fig 3 and show that the concentration is proportional to the Tyndall beam up to 3 apparent foot-candles.

The Tyndall meter can therefore be used quantitatively to measure concentrations of aggregated albumin up to 0.05 gm per cc provided the aggregated particles have reached the critical size at which they begin to settle out.

RESULTS

Heat Denaturation — Chick and Martin (7) working with hemoglobin and egg albumin, found that heat denaturation is a reaction between protein and water which takes place at any temperature but has an extraordinarily high temperature coefficient. The velocity of the reaction is also influenced by the pH of the solution and the presence of salts. Heat coagulation involves two processes (1) the denaturation of the protein and (2) the separation of the denatured protein in flocculated form. They determined the velocity constant of the denaturation and calculated the temperature coefficient. In Fig 4(a) the logarithm of K , the velocity constant, is plotted against temperature from their results for hemoglobin. Their results for egg albumin are at a higher temperature range where the temperature coefficient is apparently increasing with increasing temperature. In order to compare heat and light denaturation with the same material isoelectric egg albumin, unirradiated, was put in baths of 40°, 50°, 60°, and 65°C. At the isoelectric point flocculation proceeds so rapidly that the rate of aggregation may be taken as a measure of the rate of denaturation. The rate of aggregation was followed by measuring the strength of the Tyndall beam and it was assumed that a given Tyndall reading indicated the same condition of aggregation in all solutions. Taking

the velocity as $1/t$ where t is the time necessary to reach a certain Tyndall beam reading one finds the results for velocity given in Table I. At 40° the reaction between protein and water proceeds so slowly that it is practically negligible. Between 50° and 60° the temperature coefficient determined by the ratio of the velocities is 14.3 for a 10° rise in temperature. In Fig 4(b) the logarithm of the velocity of heat denaturation of egg albumin is plotted against the temperature from these results.

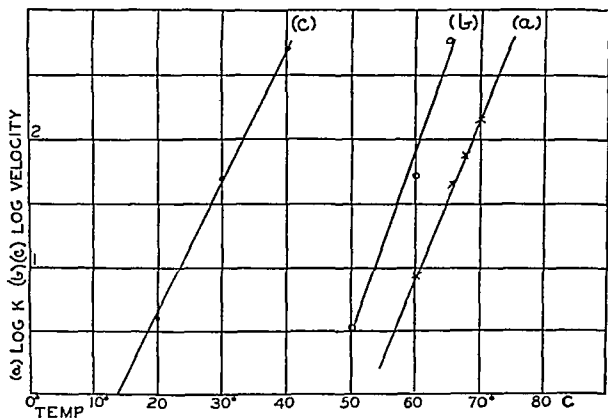


FIG 4 (a) Log K (velocity constant) plotted against temperature for heat denaturation of hemoglobin (Chick and Martin) (b) Log velocity of heat denaturation of egg albumin (c) Log velocity of flocculation after light denaturation of egg albumin

As flocculation only follows denaturation at the isoelectric point, in salt free egg albumin, solutions were heated to 90° for 10 minutes at a pH of 4.4 and 6.4 where they are denatured without flocculation. After heating they were cooled to 4° and then brought to the isoelectric point. There was immediate flocculation at this temperature showing that after heat denaturation the subsequent flocculation, although dependent on pH, occurs at a low temperature.

Denaturation by Ultraviolet Radiation—Isoelectric egg albumin (pH 4.8) exposed to ultraviolet radiation in quartz tubes, 6 inches from a quartz mercury Uviarc, and kept at a temperature of 4°C during radiation, showed only a trace of opalescence after 30 minutes radiation. When solutions radiated in this way were subsequently put in a constant temperature bath at 40°C they became opalescent and the rate of increase in opalescence, determined by Tyndall beam readings, is shown in Fig. 5. The Tyndall readings reached a practically constant value at the end of 1½ to 2 hours. By this time the flocculated material was settling out so the critical particle size had been reached and the Tyndall beam reading 2 hours after immersion in a 40° bath was taken as a measure of the amount of material denatured by the radiation as unirradiated albumin does not flocculate at an appreciable

TABLE I
Velocity of Heat Denaturation of Egg Albumin

Temperature	$t = \text{min. to reach}$ Tyndall beam = 1.0	Velocity = $1/t \times 1000$	log ₁₀ velocity
°C			
40	5760		
50	285	3.5	0.54
60	20	50.0	1.7
65	1.7	588.0	2.77

rate at 40° (see Table I). In Fig. 6 the final Tyndall beam readings are plotted against the time of radiation, and these Tyndall readings give the concentration of denatured and flocculated material by comparison with Fig. 2. These results show that after denaturation with ultraviolet radiation the denatured material will flocculate rapidly at a temperature much below that necessary for rapid flocculation of unirradiated albumin.

In Figs. 5 and 6 the circles are Tyndall readings obtained after radiation at 6 inches from the arc at 14° and the crosses after radiation at 6 inches at 4°C. As the rate of flocculation after radiation and the final Tyndall beam reading reached at the end of 2 hours is the same the temperature coefficient of the light denaturation is 1. It is interesting in this connection to note that Gates (8) found a temperature coefficient of 1.02 for the inactivation of pepsin by ultraviolet radiation.

However, the process leading to flocculation after light denaturation has a high temperature coefficient. In Fig 7 the results are given for

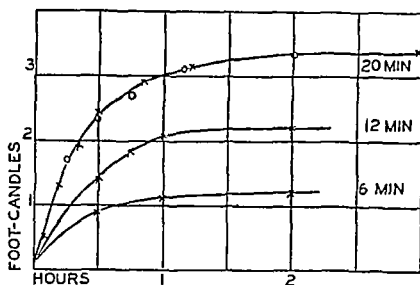


FIG 5 Increase in Tyndall beam on immersion in bath at 40°C for solutions radiated 6, 12, and 20 minutes at 6 inches from the arc (X = results for solutions radiated at 4°C and o for solutions radiated at 14°C). Ordinates = Tyndall beam in apparent foot-candles. Abscissae = time of immersion in bath.

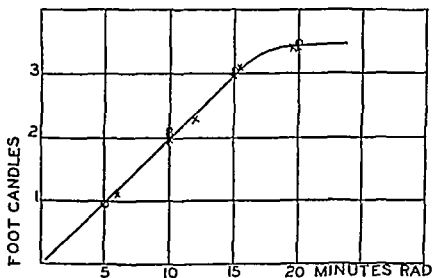


FIG 6 Final Tyndall beam readings after 2 hours in 40°C bath. Abscissae = time of radiation (X = results for solutions radiated at 4°C and o for solutions radiated at 14°C). Ordinates = Tyndall beam in apparent foot-candles.

the rate of flocculation at 20°, 30°, 40° for solutions radiated 20 minutes at 4°C at 6 inches from the arc. Some flocculation occurs even

at 5° though the rate is slow and after a week in the ice box a radiated solution showed a Tyndall reading of 0.72 apparent foot-candles. From Fig. 7 the temperature coefficient can be calculated by finding the velocity required to reach a certain degree of opalescence. In Table II the opalescence reached in a certain time is given for 20°, 30°, and 40° baths. From these results the temperature coefficient of flocculation after radiation was found to be 10.3 and in Fig. 4(c) the logarithm of the velocity to reach a certain degree of opalescence is plotted against temperature and shows, by comparison with Fig. 4(b) (unradiated albumin), that after radiation albumin flocculates

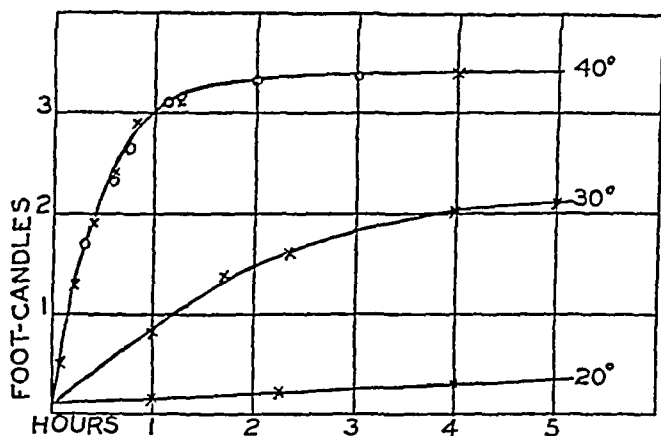


FIG. 7 Increase in Tyndall beam in solutions radiated for 20 minutes at 6 inches on immersion in baths at 20°, 30°, and 40°C (X = solutions radiated at 4°C and o = solutions radiated at 14°C). Ordinates = Tyndall beam in apparent foot-candles. Abscissae = time of immersion.

at an appreciable rate at a temperature as low as 12°, and that the rate of flocculation increases rapidly with temperature, whereas unradiated albumin shows no appreciable flocculation at temperatures below 45°C.

In coagulation by ultraviolet radiation therefore the first part of the process, the denaturation of the protein molecules, takes place at the same rate at any temperature. It has been shown in previous work (1) that it occurs over a wide pH range but the rate of light denaturation has not been investigated except at the isoelectric point and may be found to vary with pH as has been found for the photoinactivation

of pepsin (9) The subsequent flocculation of the denatured molecules only occurs at the isoelectric point in salt free albumin and although it occurs slowly at temperatures as low as 5°C, the rate of flocculation increases rapidly with temperature (temperature coefficient = 10⁺)

It has been previously noted (2, 3) that the temperature of coagulation is lowered after radiation but the process had not been studied in detail The very high temperature coefficient of heat denaturation and of flocculation after light denaturation shows that for these two processes the molecule must be put into an active state by the absorption of a large amount of energy before the process can take place

TABLE II
Flocculation after Light Denaturation

Time	T ndall readings			Tempera ture coefficient Vel 40 Vel 30	Temperature	Velocity = 1/t to reach 0.6	log ₁₀ velocity × 1000
	20	30	40				
<i>min</i>					<i>C</i>		
2			0.6	10.0			
14			1.5	9.4	40	0.5	2.7
20		0.6			30	0.05	1.7
24			2.0	11.5	20	0.004	0.6
132		1.5					
240	0.6						
272		2.0					

The high temperature coefficient of heat denaturation is thought to be associated with a chemical reaction between the protein molecule and water which is followed by the physical process of flocculation The high temperature coefficient of flocculation after light denaturation would lead one to suppose that the process is, in part at least, a chemical one and it is probable that the entire process of protein coagulation with ultraviolet radiation involves not two but three steps The first is a physical process which produces a permanent change in the molecule which we may call light denaturation It is independent of temperature and occurs over a wide pH range and unpublished observations by the author indicate that it occurs in the absence of water The second is a chemical reaction between the light denatured

molecule and water with a high temperature coefficient, which may be similar to the first step in heat denaturation but occurs at a lower temperature. The third, the flocculation of the light and heat altered molecules is a physical change similar to flocculation after heat denaturation. This conception of three steps in the process is borne out by the following observation.

A solution radiated at pH 6.4 has passed through step 1 but not steps 2 and 3. If this solution is heated to 40°C for 2 hours there is no increase in opalescence, owing to the pH, but it has now passed through step 2. If this solution is then cooled to 4°C and brought to pH 4.8 there is a rapidly developing opalescence which reaches a Tyndall beam reading of 1.95 apparent foot-candles at the end of 1½

TABLE III
Rate of Denaturation by Ultraviolet Radiation

Time of radiation	Opalescence 1.5 hrs. at 40	Concentration of denatured protein	Denaturation	C = concentration unchanged protein	Log C	k Velocity constant
hrs.		gr. per 100 cc.	per cent			
0				100	2.0	
5	1.0	0.024	21	79	1.892	
10	2.0	0.037	32	68	1.8325	0.0384
15	3.0	0.051	45	55	1.74	0.0391
20	3.4	0.057	50	50	1.699	0.0345
						0.0373

hours while a solution radiated at pH 6.4 and kept at 4°C (step 1 but not step 2) shows only a slight opalescence (0.6 foot-candles) when brought to pH 4.8. Therefore, heating to 40°C, at a pH where no flocculation occurs produces a permanent change in the light denatured molecule, a change which is necessary before the final step of flocculation can take place.

When the amount of material denatured by different lengths of exposure is calculated from the Tyndall beam readings in Fig. 6 the logarithm of the concentration of unchanged albumin plotted against time of radiation (see Table III and Fig. 8) indicates that the process of denaturation is a unimolecular one, the velocity varying with the concentration of unchanged material.

At 6 inches from the Uviarc a 1 per cent albumin solution is half denatured in 20 minutes and the denaturation is complete in about 2 hours. The Uviarc has an ultraviolet radiation intensity of 9 ZnS units a minute at 6 inches. Therefore a radiation of 20 minutes gives 180 ZnS units or approximately 1.3×10^8 ergs per sq. cm.

Velocity Constant and Critical Increment—The velocity of the second part of the coagulation process, the heat change leading to flocculation of the denatured albumin, could only be determined by the reciprocal of the time to reach a certain degree of opalescence. It is possible, however, to calculate the velocity constant of the first part of the

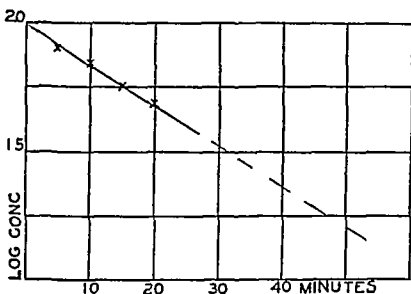


FIG 8 Log of concentration of undenatured albumin plotted against time of radiation

process, the light denaturation of the albumin molecules, and the results are given in the last column of Table III. In calculating K which is equal to $\frac{\log C - \log C_n}{t_n - t_o}$ the logarithms to base 10 are multi-

plied by 2.3 to convert them to natural logarithms. As the process of denaturation is independent of temperature and K_1 (velocity constant at absolute temperature T_1) = K (velocity constant at absolute temperature T_2) the critical increment $E = 0$ since $\log K_1 - \log K_2 = \frac{E}{R} \left(\frac{T_1 - T_2}{T_1 T_2} \right)$. In other words the protein molecule is in a condition favorable for light denaturation without addition of energy.

Heat denaturation has a very large critical increment given as 130,000 calories (10). The high temperature coefficient of the second part of the light coagulation process, the heat change leading to flocculation of the light denatured molecules, indicates a large critical increment for this step in the coagulation process.

Freezing—It has been found (11) that the proteins in muscle juice are partially denatured by very prolonged freezing at about -2°C . Solutions of isoelectric egg albumin frozen at -6°C and kept at -2°C for 3 hours showed no opalescence when thawed out and brought to 40° for 2 hours. Freezing for a period of 3 hours, therefore, does not appreciably denature isoelectric egg albumin.

CONCLUSIONS

The coagulation of isoelectric egg albumin solutions, on exposure to ultraviolet radiation, involves three distinct processes, (1) the light denaturation of the albumin molecule, (2) a reaction between the light denatured molecule and water which may be similar to heat denaturation but occurs at a lower temperature, and (3), the flocculation of the denatured molecules to form a coagulum. The light denaturation is unimolecular, independent of temperature, and occurs over a wide pH range. The reaction between the light denatured molecule and water has a temperature coefficient of 10^{+} and occurs rapidly at 40°C , a temperature at which heat denaturation is inappreciable.

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ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS IX

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I

With lines of rats well inbred it has been shown¹ that the relationship between orientation angle θ and slope of surface α in the geotropic creeping of young rats is specific and quantitatively reproducible. It is also found that the capacity of individuals to exhibit variation in geotropic response (orientation) is likewise a reproducible function of the genetic constitution of these rats. The capacity to exhibit variation of response is measured by the functional connection between relative variations of θ and the magnitudes of the performance, θ , which in turn depend upon the intensities of the exciting force due to the acting gravitational component. It was shown that the "proportionate modifiable variation" is a constant which differs for each of several lines tested. The proportionate modifiable variation is taken as the fraction of the total relative variation of performance which is dependent upon (or modified by) the intensity of excitation, and thus upon θ (Crozier and Pincus, 1931-32 *a*).

The evidence for the invariant character of the capacity to vary the performance θ , as a function of the impressed intensity of excitation, is derived from several sources. The percentage of modifiable variation is independent of the size of the sample of individuals or of measurements of θ , provided the sample is really homogeneous in this respect (Crozier and Pincus, 1931-32 *a, b*, etc.). It is also quite unaffected by experimental modifications of the curve connecting θ and α , such as are brought about by forcing the young rats to carry additional loads (Crozier and Pincus, 1931-32 *b*) or by causing them to creep more rapidly when adrenin is administered (Crozier and Pincus,

¹ Crozier and Pincus (1929-30 1931-32*a*), Crozier, 1935

1932-33 *a*) Moreover, in several pure lines which have been studied, the percentage modifiable variation of geotropic responses is the same in adult rats as in the young (Crozier and Pincus, 1931-32 *a*, 1932-33 *b*), despite the difference in appearance of the θ - α graphs, and is here likewise unaffected by the action of adrenin which markedly alters the relation of θ to α (Crozier and Pincus, 1932-33 *b*)

In hybrid young rats, obtained by crossing inbred lines, the *total* variation of performance is either increased or about the same, but the dependence of the variation of observed θ upon $\log \sin \alpha$ is definitely less, and the percentage modifiable variation is markedly decreased (Crozier and Pincus, 1929-30, Crozier, 1935) This is due to an increase, in the F_1 hybrids, of the portion of the total variation of measured performance which is not open to control as a function of the intensity of excitation Hybridization thus brings about a kind of modification of the young rats' ability to exhibit variation of θ which other attempts to modify the θ *vs* α curve do not induce

It has been pointed out that the evidence thus far available, from the study of other phenomena of excitation and response as well as from the present series of cases, supports the conclusion that the capacity to vary response is limited, under controlled conditions, by the intensity of induced excitation (*cf* Crozier, 1935) This capacity must be measured as a function of the conditions of excitation, if it is to be measured at all When this is done, the proportionate modifiable variation of geotropic performance appears to be determined by the number of appropriate excitation units open to stimulation (*cf* Crozier, 1935), and that in each pure line these are determined by the genetic constitution of the rat Consequently it is important to discover the nature of the heterosis-like behavior of the variability of performance in F_1 hybrids, the behavior of backcross individuals, which show by comparison with F_1 an expected return to the magnitudes of the variability functions characteristic of their phenotypic composition with respect to geotropic performance, suggests a purely genetic interpretation of the effect, although in certain instances (Crozier and Pincus, 1931-32 *c*) this is not sufficient as explanation It is of interest to discover whether the decreased proportionate modifiable variation in young F_1 individuals is maintained throughout their lives, or whether it is modified in the adult state The in-

terpretation will be different in the two cases. For the heterosis like phenomena appearing in the curve of mean θ vs α for F_1 individuals of the cross between rats of inbred lines A and B it was suggested that the effect could be understood by supposing that in the hybrid individuals relevant elementary developmental processes fail to "keep step" as in the pure line rats (Crozier and Pincus, 1931-32 *c*). In this event, the irregularities might be expected to disappear in the adult.

II

This matter was tested by examining the geotropic orientations of a group of 5 F_1 adult females 7 months old from the cross $A \text{ } \varnothing \times B \text{ } \sigma$ (*cf* Crozier and Pincus, 1931-32 *c*). We desired to determine the variability of θ , and its possible modification under the action of adrenin. In the adults of race A adrenin has no effect on the proportionate modifiable variation (Crozier and Pincus, 1932-33 *b*). The hybrids of $A \times B$ have the particular advantage for this test that in all essential respects the B factors for threshold and for $\Delta\theta/\Delta\alpha$ are all dominant over those of A (Crozier and Pincus, 1931-32 *c*). (In other cases, where the slope $\Delta\theta/\Delta\alpha$ in different portions of the θ vs α curve is due to factors of differing genetic origin, irregularities in the variability functions may complicate the situation. This will be discussed elsewhere.) In the cross $B \text{ } \sigma \times A \text{ } \varnothing$ the $\varnothing \varnothing$ when young appeared to show less of the "heterosis effect" than the $\varnothing \varnothing$ from the opposite cross (Crozier and Pincus, 1931-32 *c*).

For observations on orientation these rats were prepared by being fed after creeping upward upon an inclined plane, during about 10 days. The procedure was as outlined in the account of a preceding experiment with adult A rats. The determinations of the thresholds were made as follows: (1) the upper limit was determined by the fact that above $\alpha = 80^\circ$ there was marked slipping and in the case of one animal, No. 1, creeping even at 80° was impossible, (2) the lower threshold was determined in two ways: first by the direct observation of the mode of creeping: it was noted that the injected animals at 10° and at 12.5° in general crept in the "typical" manner. Typical creeping was more obvious at 12.5° than at 10° in the injected animals and the number of downward movements

We are indebted to Dr W H Stavsky for painstaking assistance in these preparations.

made was much less in the injected than in the uninjected animals. The second method involved the running of the uninjected rats at angles below 15° , it was then noted that the average value of apparent θ was much higher than at 15° . This increase in average value of θ is of course an artifact and is due to the fact that the animals tend to run away from the observer, the average θ then obtained is obviously determined by the shape of the creeping plane. It is possible at the angles below the threshold to start the animals at the top of the plane and have them run down at θ 's corresponding to those recorded. Actual measurements of downward θ 's were not taken, however, as the rather abrupt change in θ values from threshold to the below-threshold values was sufficiently significant. Below threshold, moreover, $P E_\theta$ rises suddenly to very high values.

These rats, although amenable to handling, were much more "nervous" than *K* strain adults. In consequence there was often quite a delay between successive orientations. Furthermore any loud noise coming from other parts of the building had a definite effect upon the animals. In certain cases it seemed that unknown events occurring during the day must have affected the animals, for on various evenings they exhibited inexplicable jumpiness. These disturbances appeared not to affect the magnitudes of θ during geotropic progression, if anything, their influence might be expected to increase the uncontrollable variation of performance, but the results give no such indication.

III

Determinations of mean orientation angles for adult rats of the F_1 generation $A \times B$ are summarized in Table I. The threshold slope of surface was at $\alpha = 15^\circ$. This agrees with that obtained for a series of adult *A* rats (Crozier and Pincus, 1932-33 *b*), although the orientation angle at threshold slope is lower. Such comparisons are not profitable, however, because the shape of the curve obtained with adults varies with the age of the individual. For this reason, comparisons can only be made among measurements secured with homogeneous material. It is noteworthy, nonetheless, that in this case the young $A \times B$ hybrids (Crozier and Pincus, 1931-32 *c*) also show a threshold slope of 15° , whereas experience with other lines, including line *A*, has generally demonstrated that for the adults of any one type the threshold slope of surface is lower than with the young. The important feature of the threshold response, however, is the magnitude of the orientation angle θ .

The general form of the relationship between θ and $\log \sin \alpha$ (Fig. 1), exhibiting a discontinuity in the neighborhood of $\alpha = 45^\circ$, is that

TABLE I
Geotropic Orientations (θ) of Adult Rats $F(A \times B)$

Slope α	Orientation θ_m
degrees	degrees
15	55 22 \pm 2 32
20	56 32 \pm 3 78
25	59 58 \pm 1 98
35	66 94 \pm 2 72
40	70 32 \pm 1 10
	70 37 \pm 0 83
45	70 94 \pm 1 68
55	77 68 \pm 1 22
70	79 97 \pm 0 71
80	84 10 \pm 0 56

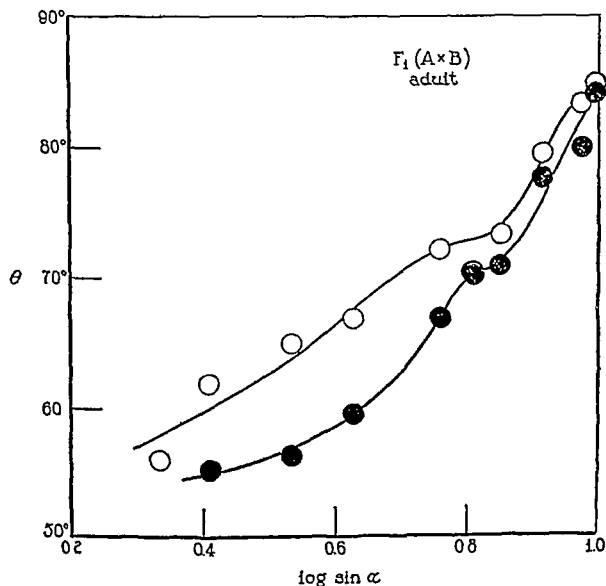


FIG 1 Mean orientation angles for geotropic progression of adult F_1 hybrid rats ($A \times B$). Lower curve, normal, upper curve after injection of adrenin 1:50,000, 1/2 ml intraperitoneally. The points do not depart from the curve by more than their probable errors.

previously observed (Crozier and Pincus, 1932-33 b)³ Complete smoothness and regularity is not to be expected in the relationship between θ and α obtained in such experiments, particularly when observations are made after injection of adrenin, or other experimental treatment. The method of progression alters as α is increased above 45° , and one cannot be sure that the time-course of the action of the drug is uniform in each instance. The important consideration is that which grows out of the fact that θ and $P E_s$ are intimately related.

The individuals involved in the measurements of Table I were also employed to test the action of adrenin. They received intraperitoneal injection of 0.5 ml of 1:50,000 adrenalin hydrochloride 1/2 to 1 hour before the observations at any one slope of surface. Rats prepared in this way were more active and crept more steadily, even at very low slopes of surface.

The five individuals tested, all females, varied in weight from 184 to 206 gm, as in all other series, there is no correlation between θ and weight, or between variation and weight.

The effect of injection with adrenin is precisely that observed with adults of race A—the curve is pulled out to the left, the threshold slope of surface is definitely lowered, but at this threshold slope the minimal response observed is statistically identical with that obtained from the un.injected rats. The data are summarized in Table II.

³von Buddenbrock (1931) suggested that in the orientation process the organism is really attempting to keep its transverse axis horizontal, the idea is of course utterly at variance with the effects of added loads. This can be tested by computing, as von Buddenbrock does, the angle δ defined by the relationship $\sin \delta = (\sin \alpha) (\cos \theta)$. According to von Buddenbrock this angle tends to be small and constant. It has been pointed out (Crozier, 1934-35) that $\sin \delta$ computed in this way is also to be regarded, in certain cases, as the proportionate measure of a force vector normal to the path of progression and parallel to the inclined surface. It was also pointed out (Crozier, 1934-35) that, far from being constant, the angle δ in fact must pass through a maximum. It is of some interest to test von Buddenbrock's idea by means of the data upon rats, since in this case especially the possible role of the bilaterally arranged statocyst mechanisms should be made clear if the inner ears do control the angle of orientation. In various series of measurements, as in Table I and Table II, δ changes systematically as α is increased, the maximum is located at $\alpha = 45^\circ$.

The effect of this concentration of adrenin is about the same, proportionately, as in adult *A* rats (Crozier and Pincus, 1932-33 *b*) The measurements are plotted in Fig 1

IV

Since the number of observations (n) at each point is not the same for the two series to be compared (*cf* Tables I and II), the values of the relative variation of θ , $(100 \text{ P.E. } \theta)/\theta$, have been multiplied by $\sqrt{n/N}$ in each case to give units independent of n and of N (*cf* Crozier and Pincus, 1929-30, 1931-32 *a*) The data giving the dependence

TABLE II

Geotropic Orientations (θ) of Adult Rats, F_1 ($A \times B$) under Action of Adrenin
 $N = 5$ $n = 45$

Slope α	Orientation θ_m
<i>degrees</i>	<i>degrees</i>
12.5	56.00 \pm 1.40
15	61.89 \pm 0.66
20	64.95 \pm 0.94
25	66.88 \pm 0.81
35	72.13 \pm 1.17
45	73.33 \pm 0.59
55	79.57 \pm 0.80
70	83.31 \pm 0.34
80	84.81 \pm 0.42

of the relative variation of θ upon θ are plotted in Fig 2 For reasons already discussed (Crozier and Pincus, 1931-32 *a*, etc), the points in Fig 2 are expected to show a progressively wider spread toward the low θ end, since each determination is subject to a standard deviation proportional to its own magnitude

It is apparent that the measures of variation for the two series, normal and adrenalized, are concordant when expressed as a function of θ This was also found for adult *A* rats (Crozier and Pincus, 1932-33 *b*) The total observed variation (*cf* Crozier and Pincus, 1931-32 *b*, etc) is 117.5 units This agrees quantitatively with the corresponding number for young rats of pure line *B*, and is much above that for young rats of race *A* (Crozier and Pincus, 1931-32 *b*), or

for adults of race *A* (Crozier and Pincus, 1931-32 *b*) It was found previously that the action of adrenin did not modify the total variation of performance in adult rats of race *A*, and this is confirmed for the adult *A* \times *B* hybrids

It has also been shown that even in cases where the total variation of geotropic performance can be modified by experimental treatments, in young rats (Crozier and Pincus, 1932-33 *a*), the percentage of the total which is modifiable as a function of θ remains unaltered (Crozier,

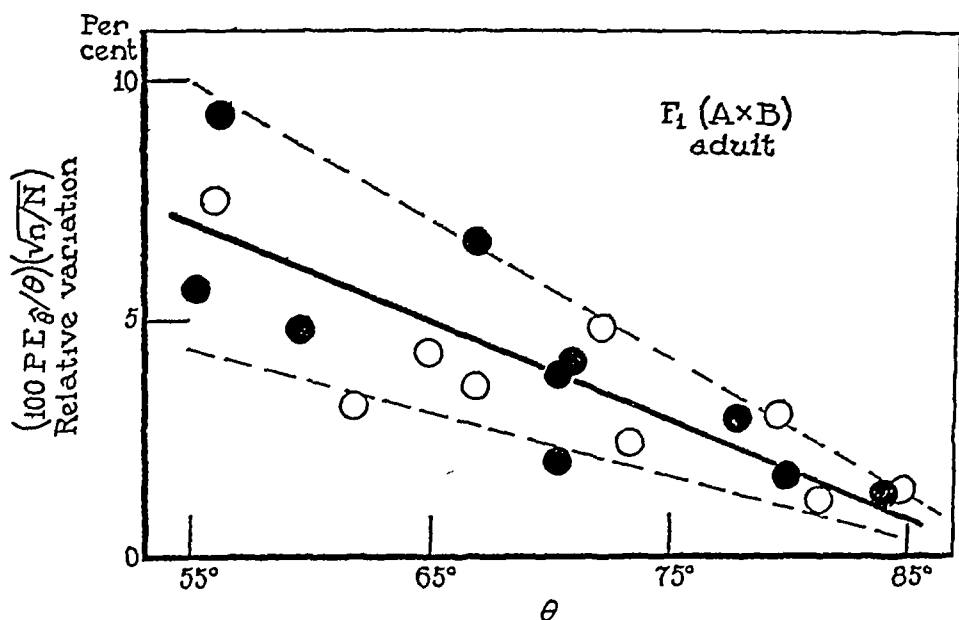


FIG 2 The relative variation of geotropic performance, corrected for N and for n , as a function of extent of orientation, for F_1 ($A \times B$) rats (see Fig 1) The variability of orientation is the same with adrenin as without

1935) In the present case the distortion of the θ vs α curve under adrenin does not affect the rat's capacity to exhibit variation of extent of orientation The percentage of the total variation which is modifiable works out to be 74 per cent This agrees precisely with the value already ascertained for young rats of race *B* (Crozier and Pincus, 1931-32 *c*)

The evidence from this experiment therefore demonstrates that the peculiar variability of geotropic performance detected in young rats obtained by crossing inbred lines tends to disappear in the course of

their development. The adult $A \times B$ rats exhibit precisely the extent and character of ability to vary geotropic performance which are specifically associated with the B line. Since in this cross the factors derived from B are dominant, this finding is entirely consistent with the view (Crozier, 1935) that the limitation of variability of performance is determined by the genetic constitution of the rat as concerns its equipment of tension receptive sensory units. It is also consistent with the idea that the disturbances of variability in young hybrids are due to the failure of relevant developmental processes to keep pace in the same orderly fashion as in the individuals of pure lines.

SUMMARY

Adult hybrid rats from the cross of races $A \times B$ show a total capacity to vary their geotropic performance which is identical with that of their B parents. The proportionate modifiable variability of geotropic orientation also agrees quantitatively with that for the B parents. These relationships are not disturbed by the action of adrenin, which leads to a distortion of the θ vs α curve. Young rats of the F_1 generation show a greater proportion of unmodifiable variation of geotropic orientation. It is pointed out that the present findings support the conclusion that the capacity of rats to exhibit variation of geotropic orientation is limited by their genetically determined composition and that the special condition in the young hybrids may be understood as due to a kind of temporary disharmony of developmental events.

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ADAPTATION OF CUTANEOUS TACTILE RECEPTORS III

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I

In a recent paper (Hoagland, 1934-35) evidence was presented indicating that adaptation to pressure as measured by the failure of nerve impulses from single mechanoreceptors in frogs' skin is produced by an inhibitory neurohumor, probably containing potassium, released from cutaneous cells when they are pressed upon. The receptors are axon endings branching among the cutaneous cells. The released potassium was assumed to raise the concentration of potassium, K , outside of the axon branches, and thus to reduce their excitabilities by lowering the ratio of potassium inside the nerve to that outside (K/K_o).

A steady pressure applied to the skin containing a sensory ending usually sets up only one or two impulses at the moment of its application and again on its release. A movement of the skin is necessary to stimulate. There is an obvious resemblance here to the stimulation of nerve by a constant current. In both of these cases we may regard the failure to respond to the continuous pressure or continuous current as a failure of excitation due to the establishment of a back E M F resulting from steady state kinetics and involving a reestablishment under the constant conditions of a new polarization level of the nerve membranes. If we choose to call this phenomenon adaptation it should be borne in mind that, while it may be interpreted in terms of the nerve's K/K_o ratio as a measure of polarization, it is not the same phenomenon as the adaptation or peripheral sensory inhibition discussed in this and in previous papers (*cf* Hoagland 1935).

In the present paper certain experiments are described which show (a) that the adaptation, or "peripheral inhibition" of the endings is *not* a product of their own activity, and (b) that the inhibition, under certain conditions, spreads through the skin for appreciable distances. The single axon cutaneous endings (*cf* Adrian, Cattell, and Hoagland, 1931) in a frog's skin were stimulated with a jet of air passed through

a nozzle and interrupted by a notched disc rotated by a motor. The axon potentials were amplified and recorded by means of a Matthews oscillograph used in conjunction with a loud speaker. Adaptation to an interrupted pressure may be regulated as to its duration in terms of the properties of the discs used to interrupt the jet (Hoagland, 1932-33*a*)¹

II

The time necessary for recovery from adaptation is 30 to 60 seconds and hence is of an order of magnitude completely different from the short intervals of a few sigma required for recovery from the refractory state in nerve. Fig. 1 shows two out of eight typical plots of the number of nerve impulses from single axon endings, set up in response to intermittent air jet stimulation, as a function of the durations of rest periods allowed between the bursts of intermittent stimuli. After aiming the nozzle at the spot the preparation was not moved. The air pressure, temperature, disc, and frequency of stimulation were constant for any one experiment.

The recovery curves are consistent with the humoral inhibitory hypothesis we have advanced and cannot be accounted for in terms of any known events occurring solely in free nerve endings independently of their environment.

Cattell and Hoagland (1931) used two nozzles (*A* and *B*) impinging air jets on the skin a few millimeters apart, to study adaptation to the separate interrupted jets. A card was inserted between the streams from the two nozzles to avoid spread of the stimulus and to make it possible to observe the effects of each jet independently of the other. Since, however, movement of the skin is the actual stimulus, it was impossible by this means to be sure that the mere limitation of the spread of the air really limited the stimulus. The areas under each

¹ Dr. Morgan Upton recently pointed out to me an interesting observation concerning the adaptation of tactile receptors in man to an interrupted repetitive stimulus. When driving a car in one's shirt sleeves the cloth of the sleeve of the arm resting on the outer open window sill of the car is often whipped by the wind against the skin. Under this treatment the skin rapidly becomes locally anesthetic to touch and pressure, only the deep pressure sensibility remaining.

nozzle were supplied by the *same* single branching axon and the nozzles were adjusted above the responsive area so that it usually took about equally long for each of them to produce adaptation in the undisturbed preparation when the same notched disc was used

It was found in these experiments that when adaptation was produced by nozzle *A* the action of nozzle *B*, applied immediately afterward, was either completely unable to produce impulses or that the number of impulses that it could produce were greatly reduced in

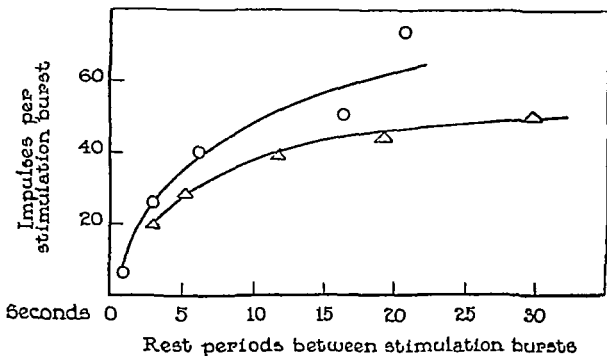


FIG 1 Two typical curves showing the total number of impulses produced by an interrupted jet when applied to each of two sensitive endings. The total number of impulses for each burst of stimuli, as counted from photographs, is plotted against the time allowed to elapse between the successive bursts of stimulation. Forty two impulses per second were used to determine the lower curve with one preparation, one hundred and two impulses per second were used with another preparation to determine the upper curve.

number. At the time, we interpreted this to mean that the antidromic spread of impulses in the axon branches from the region first stimulated to that stimulated under *B* immediately afterward, produced adaptation in the endings under *B*. In short, that the impulses themselves produced adaptation. It was, however, also possible, but from our observations at the time improbable, that either nozzle *A* or *B* might have stimulated simultaneously the areas under both, through movements of the skin.

Since a gradually and steadily increasing air pressure, which sets up no nerve impulses, nevertheless was found to produce adaptation, and since adaptation so produced at *A* did not show any inhibitory effect on the area under *B*, we concluded that adaptation may be produced either by the stimulus alone which initiates no nerve impulses or, as a result of the experiment described in the above paragraph, by nerve impulses backfired over the branching axon into an unstimulated region. It did not occur to us to consider the possible effects of a spreading inhibitory humor released from the skin itself.

The following experiments indicate that we were incorrect in our suggestion that nerve impulses produce adaptation of the tactile endings.

The time of adaptation to an air jet stimulating forty-five times per second was measured by listening to the impulses on the loud speaker. After a recovery period (requiring 30 to 60 seconds for different endings) electrical stimulation was applied over the recording electrodes without moving the preparation. Forty-five "make" and "break" shocks per second from a Harvard coil were delivered to the nerve at a shock intensity sufficient to insure stimulation of all of the fibers in the nerve trunk. The stimulation was maintained for a period of from five to ten times as long as that previously found necessary to produce complete adaptation by the forty-five air puff stimulations delivered per second from the nozzle. Immediately on cessation of the electrical stimulation a switch was closed engaging the stimulating electrodes with the recording system, and the air jet stimulus of forty-five puffs per second was at once reapplied to the skin. All of some dozen preparations so tested were found to be completely *unadapted* after the electrical stimulation. A full response was elicited by the interrupted jet which was of entirely normal duration. This shows clearly that the nerve activity itself, including the activity of the ending, due to the centrifugal impulses electrically initiated, does not produce adaptation.²

² Elsewhere it has been suggested (Hoagland, 1935) that the adaptation of muscle spindle receptors in the presence of a constant stretch of the muscle may be brought about by potassium released from the contracting muscle cells and accumulating around the axon endings. The extensive experimental results of Matthews' studies (1931*a*, 1931*b*) are consistent with this hypothesis. He

The results of the two nozzle experiment of Cattell and Hoagland can probably best be accounted for in terms of a spread of inhibition from the stimulated region under *A* to that under *B*, 2 or 3 mm away, as was recently suggested (Hoagland, 1934-35). But if this is true it at first seems puzzling that there should be no spread of inhibition to an area under *B* when the jet through *A* increases gradually, and fully adapts the part of the ending in the area under it (Cattell and Hoagland, 1931). The experiments to be described next indicate that the vibratory movement of the skin, such as is produced by the interrupted jet, is an important factor in spreading the inhibition for appreciable distances.

If a piece of skin is excised containing many freely branching axons and if impulses are recorded from the attached common nerve trunk in response to constant steady pressure from a glass plate, the endings become adapted temporarily and fail to respond to stimulation of the skin. Under these circumstances recovery in some of the endings manifests itself in 10 seconds and seems to be complete in all of them in from 2 to 3 minutes. There is, however, no observable spread of the adaptation produced by the constant pressure to areas immediately adjacent to the stimulated region. A modification of this experiment was performed with the single fiber preparation by pinching the skin containing it with forceps and thus producing adaptation. Pinching the skin immediately *adjacent* to the area containing the single ending did not produce adaptation.

That actual spread of adaptation from *A* to *B*, where an interrupted stimulus is used, does occur may be seen from the following typical protocols.

Nozzle *A* was focused on a sensitive spot, *1 c*, a spot giving an appreciable duration of discharge to intermittent stimulation. Nozzle *B*, of the same bore and supplied from the same air source as nozzle *A* was directed so that its jet impinged on the skin 3 mm from that of *A*. Nozzle *B* was thus focused just off the spot so that its interrupted jet initiated no nerve impulses.

found for example, that potassium hastens the adaptation to a greater degree than other ions and that antidromic impulses backfired into the ending do not produce adaptation.

To be certain that these conditions were fulfilled, adaptation times to nozzles *A* and *B* were measured alternately by timing the duration of the discharge with a stop-watch. 60 seconds of rest were allowed between each successive determination. The following table shows a typical experiment. The zeroes under *B* indicate that no nerve impulses were produced by the nozzle stimulating at *B*.

	<i>A</i>		<i>B</i>		<i>A</i>		<i>B</i>		<i>A</i>		<i>B</i>		<i>A</i>		<i>B</i>	
Adaptation time, sec	3	0	0	0	3	2	0	0	2	8	0	0	3	0	0	0

Having thus determined the effects of the two rigidly clamped nozzles, 40 seconds of rest were allowed and then nozzle *B* was turned on and allowed to stimulate for 20 seconds. Nozzle *A*, applied *immediately after* turning off *B*, was then found to be completely ineffectual as a stimulus, *i.e.* no impulses were set up, so that the adaptation time under *A* was also zero. This experiment could be repeated many times on the same ending and is typical of eight preparations tested.

We see, therefore, that an inhibitory effect must have spread for 2 to 3 mm from the insensitive skin area under *B* to the normally responsive area under *A*. The recovery time for the ending under *A*, inhibited by stimulating the skin under *B*, was of roughly the same order of magnitude as was that for the normal time of recovery of the ending under *A* from adaptation.

From these results it appears that the spread of inhibition for a distance of several millimeters takes place only if the skin is vigorously churned by the interrupted stimulus. It is possible that the vibratory movement hastens the spread of the inhibiting humor. Unaided diffusion would normally be much too slow to account for the effects.³

³ It has been recorded elsewhere (Hoagland, 1932-33 *b*) that a spread of operational injury occurs along the lateral-line receptors causing a progressive silencing of the spontaneous discharge of the neuromasts. This depressing action spreads in some 10 minutes for a distance of 2-3 cm, decreasing with the distance from the incision. Such a rate of spread is, of course, not to be accounted for on the grounds of simple diffusion of an inhibitory humor along the lateral-line canal. Recent evidence indicates that the hair cells of the neuromasts of the lateral-line canal normally beat as cilia (Smith, 1930, *cf.* Hoagland, 1935) and are able to move colored substance through the canal at speeds of an order of magnitude consistent

In the single ending preparations tested the circulation was intact during the experiments as could easily be seen by subsequent microscopic examinations. Since Cattell and Hoagland had found no spread of inhibition from the sensitive area under *A* to the sensitive area under *B* when *A* was inhibited by a gradually increasing *uninterrupted* stimulus it seemed unlikely that the circulation was a determining factor in spreading a released inhibitory agent. To test this further, the experiments with two nozzles one focused on the sensitive spot and the other just off it, were repeated several times with frogs in which the circulation had been destroyed. The results were the same as those we have just described, and indicate that the spread of adaptation is not due to the circulation.

SUMMARY

Further experimental evidence is presented indicating that the peripheral inhibitory phenomenon known as sensory adaptation, as it is manifested in tactile receptors in frogs' skin, is produced by a neurohumor released by non nervous cells of the skin when they are pressed upon.

1 Adaptation is not produced by electrically initiated antidromic impulses backfired into the axon branches.

2 Intermittent air jet stimulation of a region of skin several millimeters distant from a responsive single ending produces failure of response of the ending to a similar direct intermittent stimulus applied to the skin containing the ending immediately afterward.

3 Constant pressure causes an ending to adapt but no spread of the effect, as described in the above paragraph, is found. This implies that the spread is the result of the vibratory movement of the skin.

4 The time curves of recovery from adaptation are inconsistent with any known properties of *isolated* nerve.

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with the observed inhibitory effects. It is possible that potassium released from broken cutaneous and muscle cells by the operation produces the inhibition of the spontaneous neuromast discharge.

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THE DARK ADAPTATION OF THE EYE OF THE HONEY BLEE

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I

The increase of sensibility to light of organisms which are kept in darkness is well known and quantitative descriptions of dark adaptation have been made for vertebrates and invertebrates. Data are plentiful for the dark adaptation of the human eye and have been treated analytically (Aubert, 1865, Best, 1910, Hecht, 1919-20, 1921-22, 1922-23, 1926-27, 1934a, Kohlrausch, 1922, 1931, Lasareff, 1914, 1926, Piper, 1903, and Putter, 1918, 1920). For lower vertebrates we find data for the eye of the chick (Honigman, 1921) and the tadpole (Obreshkove, 1921). Similar studies are those on the tuni cate *Ciona intestinalis* and the lamellibranchs *Pholas dactylus* and *Mya arenaria* (Hecht, 1926-27). In all cases the course of dark adaptation could be followed and conclusions drawn as to the velocity and range of the photosensory process.

For invertebrates other than those just mentioned only few data are available. By means of the change of phototropic response, light and dark adaptation were followed in the gastropod *Agriolimax* and quantitative data for the change of sensibility with time were obtained (Wolf and Crozier, 1927-28, Crozier and Wolf, 1928-29). For arthropods, data are only available for the eye of *Limulus* (Hartline, 1929-30), for which the electrical response in the optic nerve was taken as a measure of the change in sensitivity and for a variety of insects where tropistic changes were taken as a measure of adaptation (Dolley, 1929, von Buddenbrock and Schulz, 1933). Much information has been accumulated concerning the pigment migration in the compound eyes of arthropods during dark adaptation (for reference see Parker, 1932), but no quantitative relations between pigment migration and the photochemical changes within the eye were established.

For the study of dark adaptation of an insect eye the honey bee was chosen. A body of precise information is already available for the visual capacity of the bee's eye (Hecht and Wolf, 1928-29, Wolf, 1932-33 *a, b*, 1933-34).

II

The reaction of the honey bee to moving stripes in its visual field has been applied successfully to studies on visual acuity, intensity discrimination, and critical flicker frequency (Hecht and Wolf, 1928-29, Wolf, 1932-33 *a, b*, 1933-34). The method permits the determination of threshold values for light intensities at which the bee just gives a noticeable response. Originally the same method was applied to study of the course of dark adaptation. It was found, however, that the speed with which determinations of threshold values for light intensities could be made was not fast enough to give an adequate determination of the course of dark adaptation. A more certain way of testing had to be developed. If a bee is tied down so that it can make only head movements, the bee shows a very definite response to the slightest motion of a stripe system in front of the eye by a co-ordinated movement of the antennae *against* the direction in which the stripes are moved. The antennae might be quiet or moving, but as soon as the stripes are shifted to the right the antennae are seen at once to take a definite almost rigid position. The left antenna points at an angle of 90° to the axis of the bee's body, while the right antenna is extended straight forward, the two thus including an angle of 90° . As soon as the motion of the pattern is reversed, the antennae take a reverse position, *i.e.*, the right is extended at 90° to the body axis and the left points straight forward. This reaction occurs with great certainty and each time with the reversal of the motion of the pattern. This index response has great advantages over the one previously used for visual tests with the honey bee, because threshold determinations can be made rather rapidly, this is important for the study of dark adaptation.

The bees used for experimentation were taken from our own colonies and brought into the laboratory. The wings are clipped under slight anesthesia. After the animals were fully recovered they were fitted into glass tubes about 5 cm long with their heads sticking through a thin rubber membrane. In this position they can move their heads freely. The tube is held in position in front of a striped pattern (Fig. 1). In the tubes the bees will live for days. They are fed several times a day and take food readily.

Before dark adaptation tests are made the animals have to be fully light adapted. On account of the fact that the ommatidia of each eye include a very great area of the head and point in different directions, it is important that each element obtains a sufficient exposure to light. In case not all elements are equally well adapted to light it might easily happen that one set of elements which was well adapted would not arouse a response at a given testing intensity while some others which had not the necessary exposure to light might cause the reaction. For

adaptation, therefore, a box of opal glass was constructed $30 \times 30 \times 20$ cm with a 150 watt bulb outside of each surface. The illumination at the center of the box was 142 millilamberts and was sufficiently uniform to secure an equal exposure of all the elements of the eye to light. On account of the strong illumination outside the box precautions have to be taken that the temperature within the box is kept constant, because any rise in temperature would doubtless influence the velocity of the process of dark adaptation. By ventilating the adapting box properly the temperature was kept between 27 and 28 C. This temperature corresponded within about 0.5°C to the temperature of the dark room in which the bees were

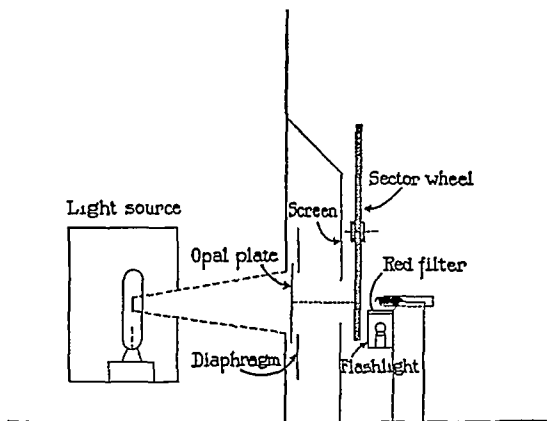


FIG 1 Diagram of apparatus for measuring threshold intensities for excitation at different periods of dark adaptation in the bee

tested. For all tests we always made it a point to keep the temperature during exposure and during test at the same level. The bees were exposed to the light after they were tied into the glass tubes. About 10 bees were light adapted simultaneously by mounting them in their tubes on a celluloid stand so that no shadows could interfere with an appropriate adaptation.

For dark adaptation test the bees were taken into a small dark room where they were exposed to a moving pattern at different intervals of dark adaptation (Fig 1). Thus determinations were made of threshold intensities at which the bees gave the first noticeable response to the moving pattern. The pattern system consists of a ground glass plate 50 cm in diameter on which 20 opaque black sectors are

pasted, leaving equally wide translucent spaces in between. The sector wheel has advantages over a system of parallel stripes because its motion can be reversed more smoothly and the bee's reaction easily followed when turning the wheel slowly around its axis to the right or to the left. Only a square area of the sector wheel, 7×7 cm, close to its periphery, is used as a test field. This area is illuminated from behind by a 200 watt concentrated-filament lamp. The amount of light coming from the source is controlled by an accurately calibrated diaphragm. With help of this diaphragm and different diffusing screens placed in front of the source, the light intensities can be varied over 4 logarithmic units.

The bee in its glass tube is placed on a stand close to the sector disc. The motion of the antennae can be seen without difficulty at high light intensities. At low intensities the antennae have to be viewed from above against a low red illumination. For this illumination a concealed flashlight bulb is used, in front of which there is a dark red filter (Wratten Filter No. 88 A) which permits only wavelengths above 6930 \AA to pass through. The longest wave-length which can be perceived by the bee's eye is about 6800 \AA (Bertholf, 1931). It therefore is perfectly safe to observe the bee's reaction to the light passing in between the sectors under these conditions. If the same filter is placed in front of the light source and the reactions of the bees studied, no response can be obtained even at the highest light intensities.

For dark adaptation tests 10 bees are adapted to light simultaneously for 15 to 20 minutes. The lights are then turned off and the bees brought into the small dark room and kept in a black box. After a certain interval the first bee is placed on the stand in front of the sector wheel, and while opening the diaphragm as quickly as possible the wheel is moved right and left until the first response of the bee can be observed. The interval of time since the animal was brought into darkness and the diaphragm reading are noted. Then the diaphragm is closed again and the next bee is tested in the same manner, and so on, until all bees which were previously light adapted have been tested. In no case was the same bee tested twice without having been fully light adapted before the new test. This was done to avoid any effect of the light to which the bee was exposed during the first test on the succeeding reading.

The first set of experiments was done during the fall of 1933 and a curve describing the course of dark adaptation was obtained. It was evident, however, that the method of exposure to light to secure light adaptation before test did not involve adaptation of a sufficiently large number of ommatidia to give an adequate description of the process of dark adaptation. New series of experiments were carried on in the spring of 1934 which gave better results, particularly because we knew by that time what the approximate range of intensities for the bee's reaction would be, so that the apparatus could be adjusted accordingly. Furthermore, it was evident from the first series of experiments that the accuracy of the decision as to the bee's reaction to the moving pattern at low intensities depends largely upon the state of dark adaptation of the observer. Thus the experiments had to be done by two people. While one was in darkness testing the bees the other took

down the times for dark adaptation and the diaphragm readings which were later translated into intensity values with the help of calibration curves

III

The data for the course of the bee's dark adaptation are presented graphically in Fig 2, where the logarithms of the threshold intensities

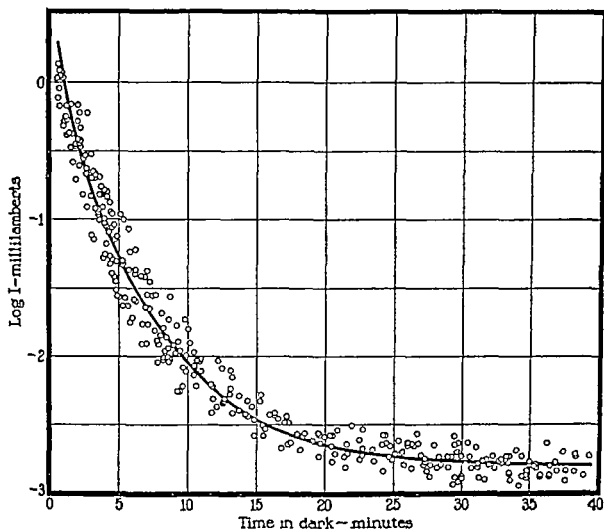


FIG 2 Relation between threshold intensities and time of dark adaptation for the eye of the honey bee

for response are plotted against the times of dark adaptation. The points fall on a smooth curve which shows that during the first few minutes in darkness the sensitivity of the bee's eye increases rapidly, then more slowly, until after about 25 to 30 minutes of dark adaptation the increase of sensitivity is minimal, so that one can assume that after 30 minutes' stay in darkness the process of adaptation is complete. The 311 points which are plotted in Fig 2 are taken from

a great number of individuals, some of them having been used only once for test, some of them used repeatedly. In case animals were used more than once they were always fully light adapted before each new exposure to the testing intensity. Since all individuals were taken from the same colony it seemed justifiable to treat the data *en masse* (cf. also Wolf, 1932-33 *a, b*, 1933-34).

The curve given in Fig. 2 shows that the dark adaptation of the eye of the bee follows a regular course. The sensitivity increases over 1000 times within about half an hour. The points plotted lie in a ribbon the width of which is apparently proportional to mean I . This indicates that the relative variation of intensity required to give the index response is constant at all levels of sensitivity during dark adaptation (cf. Wolf, 1932-33 *a, b*, Wolf and Crozier, 1932-33).

While from a mass plot, as given in Fig. 2, it is not apparent where the points determined on a single individual would lie on the curve, tests were made with a group of individuals which were numbered and used repeatedly for experimentation, so that each individual might be treated singly to see whether the curve fitted to the points would be identical with the one given in Fig. 2.

Two series of experiments were carried on. In the first case 11 animals were used and tested so many times, always with light adaptation between tests, that for each individual a sufficient number of points was obtained to fit a curve. For the second series 7 bees were used. For each series of experiments the points were plotted separately. In Fig. 3 the data obtained for the 11 animals of our first series are represented by different symbols to indicate the amount of individual variation. The curve drawn through the points is the same as that given in Fig. 2. The fit of this curve seems to be adequate for every single animal. The data for the second series of repeated tests gave the same results. Since observations were made at intervals of dark adaptation previously used, the points fall on top of the readings of the first series, to avoid confusion the data of this series are omitted in Fig. 3.

It is of interest to compare the data obtained for the dark adaptation of the eye of the honey bee with the velocity and the range of adaptation in other organisms. Among the few quantitative data available the best are those for the human eye (Hecht, 1919-20, 1921-22,

1926-27) The sensitivity of the human eye increases about 10,000 times within half an hour during dark adaptation, after which further increase in sensitivity is hardly noticeable. The same relation between time and completion of dark adaptation is found for the eye of the bee. If we compare the adaptive capacity of the human eye

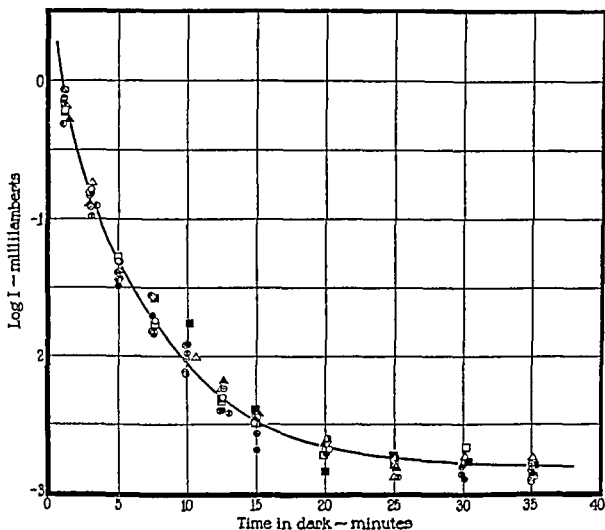


FIG 3 Relation between threshold intensities and time of dark adaptation for 11 individuals which were tested repeatedly. The threshold values for each individual are represented by different symbols.

with that of the bee we have to keep in mind that the range of dark adaptation in the human eye depends upon the number of functioning elements. The range of adaptation increases in proportion to the number of retinal elements concerned in vision. Since we cannot limit the number of elements in the bee's eye by an artificial pupil a comparison can only be made if we take the human retina as a

whole Under such circumstances we find that the range of adaptation of the human eye is 10 times greater than for the bee

If we recall that a dark adaptation curve for the human eye shows distinctly two portions, namely one for the adaptation of the cones and the other for the rods, it is noteworthy that the intensity range covered by the ommatidia of the bee's eye is pretty much the same as that which we find for the rods of the human retina

If we attempt to analyze our curves in terms of the usual equation for the chemical reactions going on during dark adaptation, we meet a good deal of difficulty From the measurements of intensity discrimination by the bee (Wolf, 1932-33 *a, b*) Hecht computed (1934 *b*) that the dark process in the bee's eye is bimolecular The curve determined in Fig 2 is definitely not second order Dark adaptation measurements should in some way reflect the fundamental nature of the dark reaction No complete theoretical relationship is at present available for the necessary connection between these two (for an approximate one, see Hecht, 1934 *a*) In this particular case the data are probably complicated by the migration of retinal pigments during dark adaptation, which would probably make the early course of the dark adaptation as measured here appear more rapid than without this complication Relevant data on the time course of pigment movement are not available At the same time, this effect, if it is involved, might also appear in the measurements of intensity discrimination and visual acuity The curve in Fig 2 is quite accurately rectilinear when $\log \log \left(\frac{I_t}{\bar{I}_f} \right)$ is plotted as a function of time in dark, where I_t is the threshold intensity at time t , and \bar{I}_f is the intensity for threshold response at complete dark adaptation, in fact, this is the curve drawn in Fig 2 No simple theory as to why this relationship is obtained has yet been forthcoming, but clearly when a complete theory becomes available it must satisfy the quantitative conditions expressed by this equation

SUMMARY

Bees which are held in a fixed position so that only head movements can be made, respond to a moving stripe system in their visual field by a characteristic motion of the antennae This reflex can be

used to measure the bee's state of photic adaptation. A curve describing the course of dark adaptation is obtained, which shows that the sensitivity of the light adapted bee's eye increases rapidly during the first few minutes in darkness, then more slowly until it reaches a maximum level after 25 to 30 minutes. The total increase in sensitivity is about 1000 fold.

The adaptive range of the human eye is about 10 times greater than for the bee's eye. The range covered by the bee's eye corresponds closely to the adapting range which is covered by the rods of the human eye.

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ELECTROKINETICS

XVII SURFACE CHARGE AND ION ANTAGONISM*

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Abramson (1)¹ made the suggestion that it would be interesting to recalculate the data of Bull and Gortner (2) on ion antagonism in such a manner as to determine the electrical charge per unit area which gives rise to the electrokinetic potential at the surface. The relation between the potential and charge for a plane surface is

$$\zeta = \frac{4\pi\sigma\lambda}{D} \quad (1)$$

where ζ is the potential, σ is the charge per cm^2 , λ is the thickness of the double layer, and D is the dielectric constant. In a sense, the charge is a more fundamental quantity than the potential. The presentation of these calculations together with others along similar lines is the purpose of this paper.

We wish to enter briefly into a discussion of the applicability of the conventional equation for the streaming potential to cellulose membranes such as were used by Bull and Gortner. Bull and Moyer (3) have discussed in some detail the relation between streaming potential and capillary size. In general, higher valence types reduce the critical radius. If the radii in these experiments are in the critical range, the mono- and divalent single salts should yield divergent values of σ and the shapes of the concentration curves would be expected to differ. As will be shown, this is not the case. The shapes of the curves are completely analogous to those obtained

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¹ p. 243

with flat surfaces (1) ² We have determined the maximum pore radius in membranes such as those employed by Bull and Gortner by measuring the minimal air pressure required to force air through the cellulose membrane, using the formula (4),

$$P = \frac{2\gamma}{r} \quad (2)$$

where P is the pressure in dynes per cm², r the radius, and γ is the interfacial tension. We found a maximal pore radius of 6.5–6.8 μ in different membranes. In addition, the mean capillary radius was found to be 0.96 μ by the method of Bull and Moyer (3). This lies above the calculated critical radius (3).

A large number of the pores are above the critical range. A number, however, are no doubt below it. It is, therefore, difficult to say exactly what the true electrokinetic potential is in such cases. On one hand we have the results of Bull (5) who found a ratio of 0.28 between the electrokinetic potential of cellulose membranes in a 2×10^{-4} N NaCl solution (as determined by the streaming potential method) and that determined by the electrophoresis of the same cellulose fibers in the same salt solution, which would indicate that the pore size in the membrane is considerably smaller than the critical size if no other factors enter in. On the other hand, Briggs (6) working with cellulose membranes of the same type as those employed by Bull and Gortner found the electrokinetic potential to be completely independent, over wide limits, of the tightness of the packing of the cellulose in the membranes. If the pore radii were in the critical range it would seem certain that correspondingly wide variations in the electrokinetic potential would have been observed. Since the membranes of Bull and Gortner were of the same type as Briggs', this indicates that their membranes were also above the critical range. For a more complete discussion of capillary size see (3). In any case, since all the measurements of Bull and Gortner were rigorously conducted under the same conditions the results have, at least, a relative significance and the data presented in this paper are to be considered in that light. For our present purpose this is sufficient.

² Chapters VI–VIII

Calculations

The following equation (1)³ was used for the calculation of the surface charge

$$\sigma = \sqrt{\frac{NDkT}{2000\pi}} \sqrt{\sum c_i \left(e^{-z_i \frac{e\zeta}{kT}} - 1 \right) + \sum c_j \left(e^{+j \frac{e\zeta}{kT}} - 1 \right)} \quad (3)$$

where N is Avogadro's number, D is the dielectric constant (7), k Boltzmann's constant, T the absolute temperature (298° K), c_i and c_j the concentrations of the cations and anions in mols per liter, z_i and z_j the valences of the cations and anions, and σ has the same sign as ζ . This is a perfectly general equation for all types of electrolytes but is valid only for plane surfaces. Pores above the critical range can undoubtedly be considered as plane surfaces. If one is dealing with a uni univalent electrolyte the above equation reduces to

$$\sigma = 2 \sqrt{\frac{NDkT}{2000\pi}} \sqrt{c} \sinh \frac{e\zeta}{2kT} \quad (4)$$

The results of these calculations are shown in Figs 1-5. In all cases the charge shown (σ) is that obtained by subtracting the initial charge in distilled water (σ_W), which is due largely to carbonic acid, from the calculated charge (σ_T). The concentration of HCO_3^- was taken as 2×10^{-6} N (1).⁴

Fig 1 shows the rapid increase of the charge on the negative surface with the addition of potassium salts of various anions. Since the cation is the same, the different behavior should be due to the anions. The trivalent PO_4^{--} shows the largest effect, the divalent CO_3^{--} and SO_4^{--} next, and the monovalent Cl^- least effect. The curves have the appearance of adsorption isotherms. Saturation has not been achieved. In this connection Moyer (8) was able to show that the charge on cholesterol particles in suspension followed Langmuir's adsorption isotherm when the charge was graphed against the OH^- concentration. The data used in our calculations are from the paper by Bull and Gortner (9), who calculated the surface charge

³ p 110

⁴ p 133

at that time by an entirely different technique and whose results are in essential agreement with those reported here

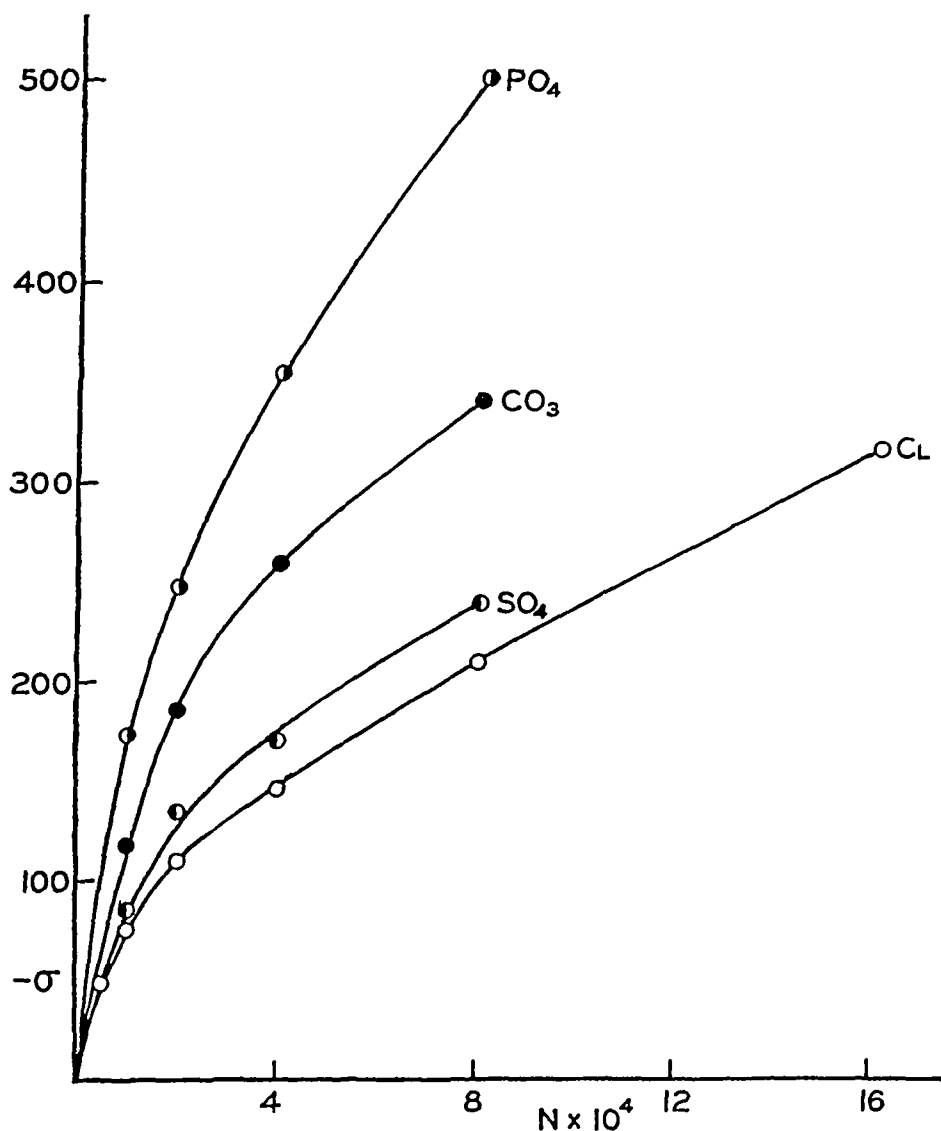


FIG 1 The electric charge density of a cellulose surface in various potassium salt solutions

Fig 2 shows the calculations for ThCl_4 , the data being taken from the paper by Bull and Gortner (9). Again the result is in essential agreement with that obtained by these two workers. This case is

interesting because an actual reversal of the sign of the charge is encountered. The curve has the shape predicted by Müller (10)

Fig 3 shows the variation of the charge with temperature, using 1×10^{-4} N NaCl as the electrolyte. The data for the calculations

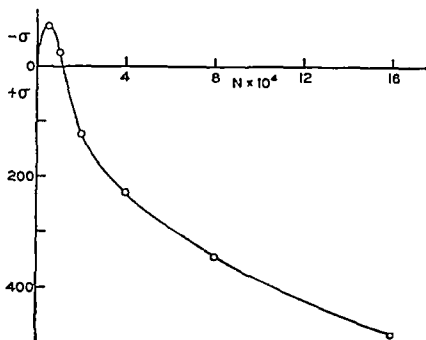


FIG 2 The electric charge density of a cellulose surface in various concentrations of ThCl_4 .

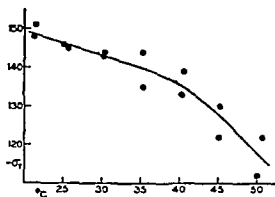


FIG 3 Variation in charge density of a cellulose surface with temperature, the electrolyte concentration was held constant

are taken from the paper by Bull and Gortner (11) together with some additional unpublished data of the above authors. Since in these data no measurements for the calculation of σ_H are available, all data are calculated as σ_T . The difference between σ and σ_T is, however, constant and very small for such high values of σ_T . Fig

4 shows the graph of $\ln \sigma$ against $1/T$. The slope of such a curve is equal to $-\Delta H/R$ (by the van't Hoff equation), provided σ is a measure of the equilibrium constant of adsorption, where ΔH is the heat of the adsorption reaction and R is the gas constant. When plotted by the method of least squares, two straight lines are obtained giving heats of adsorption of -2870 calories and -846 calories. The break comes at 39° . In this connection, it is interesting that the specific heat of water shows a rather sharp minimum at 37.5° . Inasmuch as the adsorption of ions probably involves the desorption of water, the break in the heat of adsorption of the charge is probably due

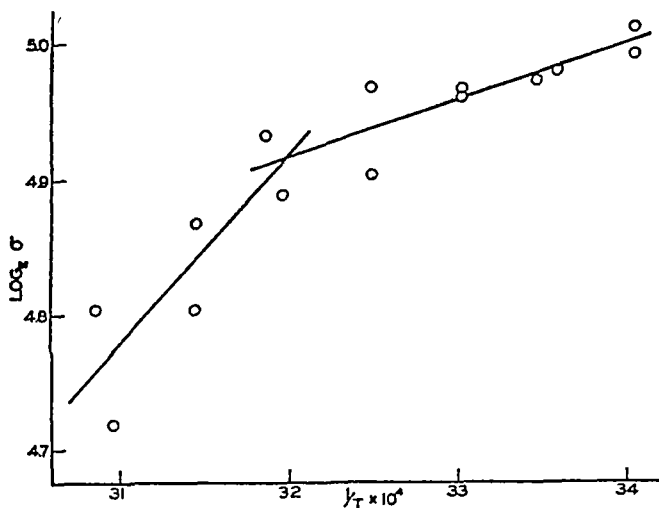


FIG 4 Illustrating the break in the curve at 39°C when $\ln \sigma$ is plotted against the reciprocal of the absolute temperature

to the change in the heat of desorption of water at this point. It is interesting that λ (the reciprocal of the thickness of the double layer) was found to remain constant or to increase with increasing temperature. This is a somewhat unexpected result since one might expect that the increased kinetic energy of the ions at higher temperatures would result in a greater thickness. The explanation is no doubt to be found in the smaller dielectric constant at higher temperatures, thus resulting in an increased force of attraction between the ions and the charged wall.

The variation of the charge with the addition of chlorides of various

cations and combinations of these salts in 1:1 ratios is shown in Fig. 5. The concentration is given in terms of anion equivalency but other methods of plotting give the same result. The data are taken from the paper by Bull and Gortner (2). The MgCl_2 curve was repeated

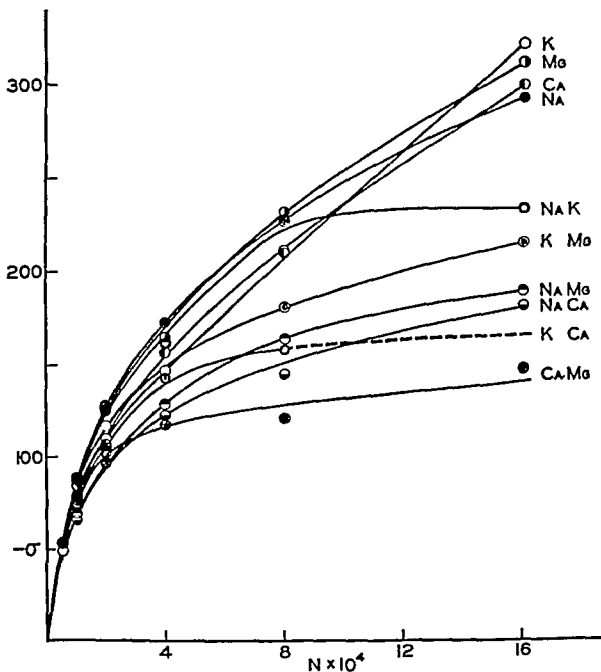


FIG. 5. Charge density and ionic antagonism. All salts are chlorides.

because of a disagreement between the unpublished results of Briggs and those of the above authors. The new data for MgCl_2 have been used here. These data agree closely with the CaCl_2 data. As can be seen, the charges produced by the single salts rise much higher than

those for the salt mixtures. Here is a completely different picture from that shown by a plot of the electrokinetic potentials against concentration (2). Unquestionably, ion antagonism is exhibited and the effect increases in the higher concentrations where our calculations are most accurate. This, to our knowledge, is the first time ion antagonism has been directly traced to electrical effects. Evidence is not lacking to confirm the conclusion. There is, for example, a marked ion antagonism between LiCl and MgCl_2 , BaCl_2 , AlCl_3 , and CeCl_3 in the flocculation of As_2S_3 sols (12). It is known that the surface charge is very important in the stability of these sols. Ion antagonism has also been observed in the coagulation of proteins (12). It is interesting to speculate on the reason why the charge with the salt mixtures is lower than that with single salts. It seems probable that in the case of salt mixtures the cations are adsorbed to a certain extent independently of one another, thus resulting in a higher cation adsorption⁵ while the chloride adsorption is much the same in the two cases, this, of course, results in a smaller net negative charge. This seems to be borne out by the fact that the charge decreases as the cation mixture is changed, in the order uni-univalent > divalent—univalent > di-divalent. It is difficult to interpret this ion antagonism in terms of ion antagonism in biological systems as the concentrations in biological systems are so uncertain. It is not impossible, however, that the two are of the same nature.

SUMMARY

1 The question of the critical pore diameter for streaming potential is discussed

2 The surface charge is calculated for cellulose in contact with solutions of K_3PO_4 , K_2CO_3 , K_2SO_4 , KCl , and ThCl_4

3 The surface charge of cellulose in contact with a solution of $2 \times 10^{-4} \text{ N NaCl}$ is calculated as a function of temperature and is found to show a sharp break at 39° . This is interpreted in terms of the change of the specific heat of water

4 A marked ion antagonism is found in NaCl KCl , KCl MgCl_2 ,

⁵ Since each ion follows its own adsorption isotherm, halving the concentration of each produces a proportionately greater total adsorption

NaCl MgCl₂, NaCl CaCl₂, KCl CaCl₂, CaCl MgCl₂ mixtures when the surface charge is calculated as a function of concentration

We wish to express our appreciation to Prof R A Gortner for his help and encouragement throughout the course of this work

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ON THE RELATION BETWEEN LITTER SIZE, BIRTH WEIGHT, AND RATE OF GROWTH, IN MICE

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I

It was shown previously that the relationship between size of litter and average weight of litter at birth, in several species of multiparous mammals, may be represented by the equation

$$W = N^K + \text{const} \quad (1)$$

where W is the average birth weight of a litter, N is the number in the litter, and K is a constant (Enzmann and Crozier, 1934-35) Since $W = w \cdot N$, where w is the mean weight of a single new born individual, it is obvious that the relation between N and w is of the form

$$w = N^{-k} + \text{const.} \quad (2)$$

The (somewhat unfortunately named) "heterogonic" relationship (Huxley, 1932) in growth rates of structurally associated parts of an organic system, otherwise referred to by the (even more unfortunate) name "dysharmonic" growth (Teissier, 1934), which it certainly does not signify, is of very general occurrence (Huxley, 1932, Teissier, 1934, Needham, 1934) It is of particular interest in its bearing upon the general theory of curves of growth, and this in a manner the consequences of which seem not to have been thoroughly appreciated (*cf* Crozier, 1926-27) It is from this standpoint, in part, that we have been anxious to test experimentally the possible meaning of constancy of relative growth rates in an organic system The most convenient situation in which to do this is provided by the intrauterine development of litters of various numbers in diverse species of multiparous mammals The interpretation given for the adequacy of equation

(1) was (Enzmann and Crozier, 1934-35) that the presence of each additional fetus in a litter called forth, in the average, a proportionate fractional increase in the nutritive level of the mother, the total nutritive material available to the young being shared equally among them. This is the reasonable deduction from the fact that, when equation (1) is adequate,

$$\Delta W/\Delta N = KW/N,$$

or, the increase in weight of litter (at birth) per unit increment of number in the litter is directly proportional to the weight and inversely proportional to the number in the litter. The exponent K is then expected to have the properties of a partition coefficient. It was pointed out (Enzmann and Crozier, 1934-35) that one such important property it does exhibit, since K turns out to be essentially independent of the *kind* of mammal and of the typical mean mass of its newborn, it is thus highly non-specific. It has a magnitude $(\Delta \log W)/(\Delta \log N = 1.0) = 0.80$ to 0.90 in various series of measurements, the range is less when the value of K is obtained from the most homogeneous material, and its "best" value may be given as 0.84 .

The exponent k in equation (2) has a rather different meaning, and its size depends upon the mean birth weight of a single individual, which is comparatively specific, in genetically homogeneous material it may be expected to be even more definitely strain specific.

To extend the observational basis for these considerations we have collected further birth weights of unfed new-born mice of the Bagg albino strain, from a line inbred by brother-sister mating in this Laboratory for over 60 generations. This is the strain involved in our previous observations. Litters containing stillborn young, or in which some of the young had been destroyed by the mother, were rejected, 181 suitable first litters were obtained from mothers less than 30 weeks old. The litters were weighed as a whole, and the mean individual birth weights computed.

To test the two aspects of the interpretation suggested, the weights of the mothers were also recorded and the subsequent growth in weight of the young was measured. It is to be presumed, on the basis of theory, that the supposed increase in nutritional provisioning by the mother, as result of carrying additional young, is shared

between the mother and these young. And the subsequent growth of the young from litters of various sizes may be expected to be suggestive for an understanding of the control of growth.

II

The result of this determination of the relation between W and N is given in Table I. In Fig. 1 the data are compared with those of our earlier series (Enzmann and Crozier, 1934-35) on litters of the same strain. The agreement is satisfactorily close, although W

TABLE I

Litter size and average weight of whole litter, Baggett albino mice, second series, first litters of mothers less than 30 weeks old

Litter size N	No. of litters	Litter weight W
1	1	(1.63)
2	3	3.07 ± 0.37
3	6	4.49 ± 0.07
4	10	5.75 ± 0.01
5	26	6.28 ± 0.09
6	31	8.09 ± 0.09
7	28	9.25 ± 0.09
8	34	10.09 ± 0.11
9	15	11.30 ± 0.09
10	11	12.05 ± 0.13
11	4	13.10 ± 0.047
12	6	14.60 ± 0.089
13	1	(14.91)

tends to be consistently a little higher in the first series. The deviation observable with very small litters, and, less clearly, with very large litters, we have already commented on (Enzmann and Crozier, 1934-35). The present data are more homogeneous, from several standpoints, than those commonly available for testing the adequacy of equation (1). The slope constant in Fig. 1 is a recoverable constant in repetitive experiments with the same strain. The intercept on the $\log N = 0$ axis gives the ideal weight of 1 individual constituting a litter of 1, and thus developed in the absence of the effects of other young in the same litter. This is a little higher for our first series than for the second. It is to be presumed in general

that this weight may exhibit modifications as a function of age of mother, inbreeding, litter rank, and nutritional state. The general care of the animals in the present series was better than in the first series.

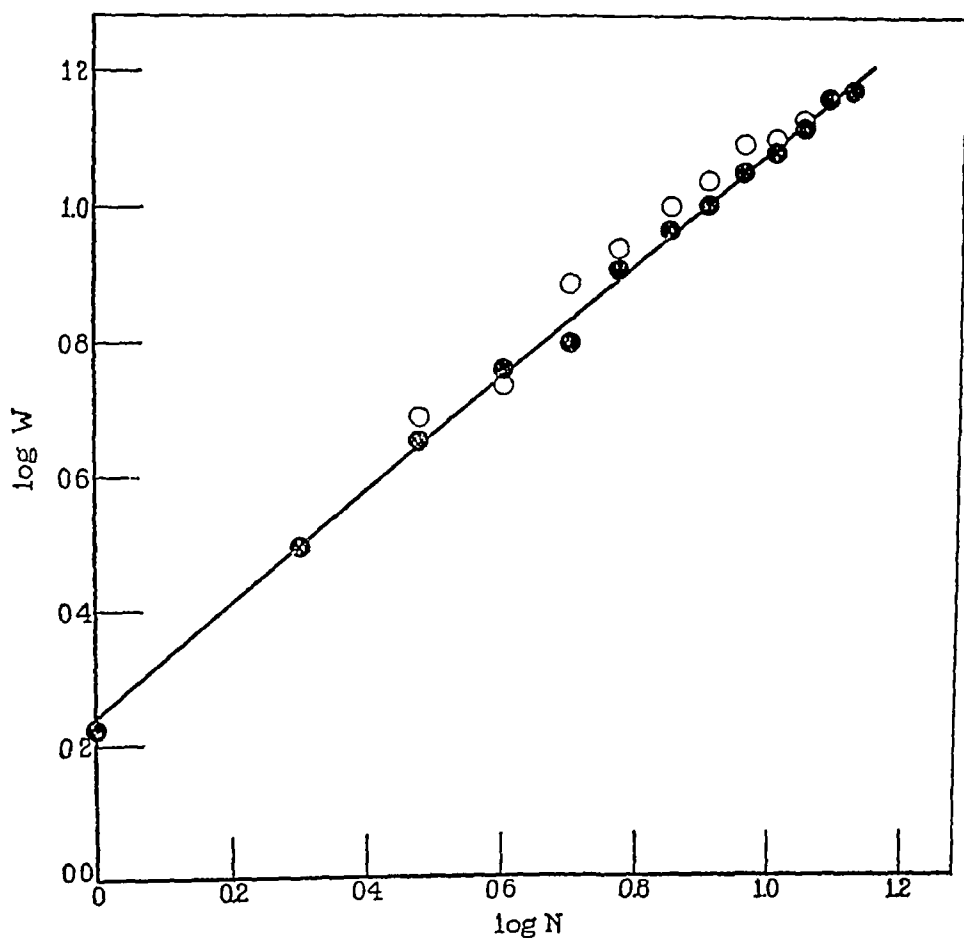


FIG 1 Weight of a litter of mice as a function of the size of the litter (Table I) (The open circlets refer to data given in a preceding paper Enzmann and Crozier, 1934-35, see text)

It is worth while illustrating the really stable character of constant K . The mean birth weight of mice from small litters is about 1.5 gm, the same value of K (0.87) is obtained from observations on pigs, with a birth weight of about 2,000 gm (Fig 2)

Fig 3 shows the relationship between w , the individual average

weight, and N . This plot is of course more apparently sensitive to variation in w at fixed N than is Fig 1. Data from diverse series of

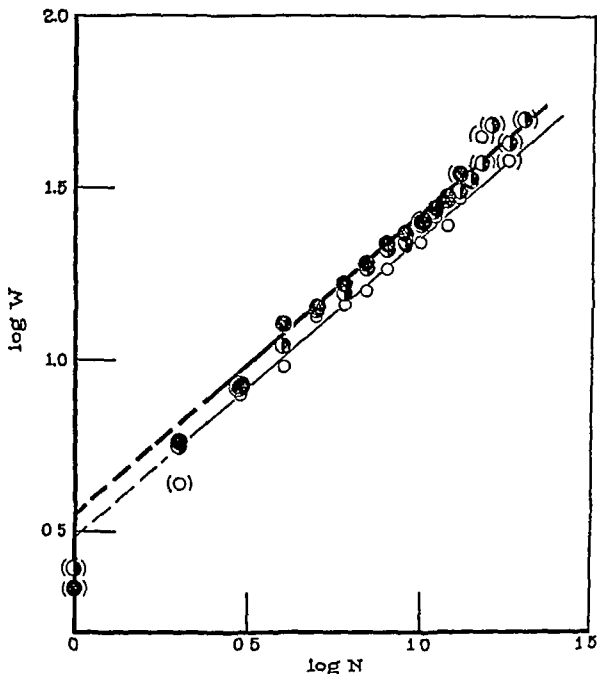


FIG 2 Data from three series of measurements of the relation between N and W for pigs, cf Lush Hetzer, and Culbertson (1934). $K \approx 0.85$ to 0.87 . The points in parenthesis are based upon less than 5 litters, w_1 differs from one stock to another, hence k differs also, in proportion to $\log w_1$.

measurements show that, empirically, the slope constant (exponent) in equation (2) may be more or less type specific, although the $\log w$ intercept is certainly very clearly strain specific, for other mammals

this is not observed. From the standpoint of the present analysis there are two quantities which should be kept clearly in view as perhaps subject to independent genetic use. One is the "ideal weight at birth of a litter of 1"— w_1 , the $\log W$ (or $\log w$) intercept. The other is the constant k , which expresses the manner in which $\Delta w/w$ is related to $1/N$. The evidence shows that both w_1 and k may be determined by genetic constitution. The estimated values of w_1

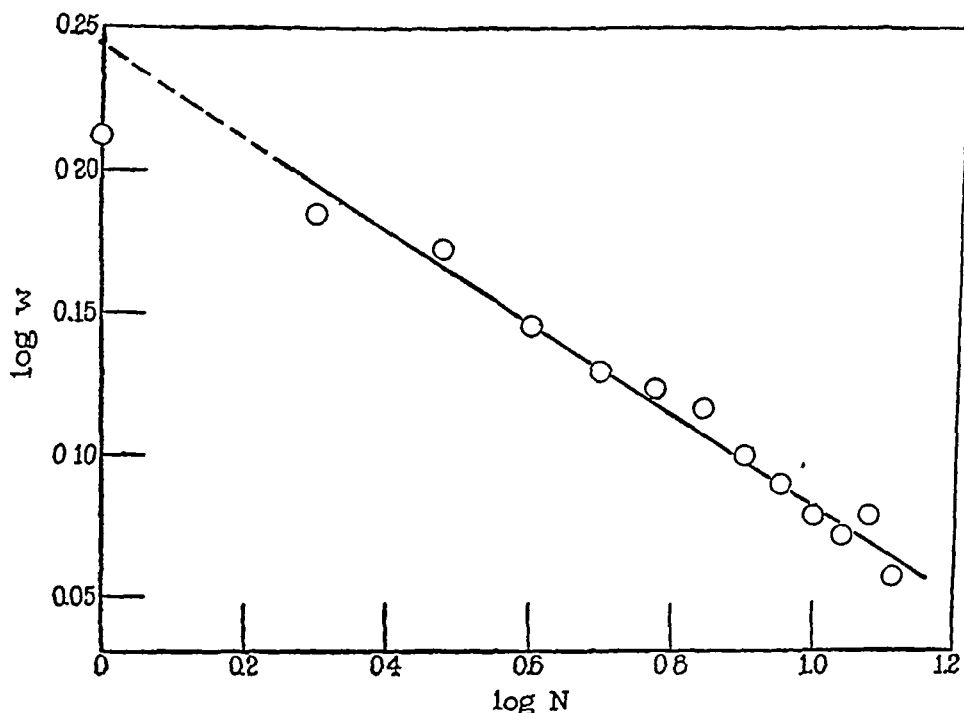


FIG. 3 The relation between average weight of one mouse at birth and the size of the litter (Tables I, II)

should give much more useful data for genetic purposes than the "average weight at birth". It is simpler and more reliable to obtain w_1 from plots such as that given in Fig. 1, but the constant k may also have its uses in comparing strains. k and w_1 are of course interrelated, k is directly proportional to $\log w_1$, since, from (1) and (2),

$$k (\Delta W/W) = -K (\Delta w/w)$$

$$\log (Nw) = -(K/k) (\log w) + \text{const}$$

$$k = - \frac{(K-1) \log w}{\log N - \text{const}},$$

and

$$k = \frac{(K-1) \log w_1}{\text{const.}} \text{ when } N = 1$$

We have already seen (Enzmann and Crozier, 1934-35) that K is practically non specific, hence k is determined by its proportionality to $\log w_1$. Studies on the inheritance of birth weights are rarely recorded in such a way that w_1 may be estimated from the data, but it is clear that this procedure should be followed, as the tabulated mean weights are obviously influenced by heterosis phenomena and by factors affecting fertility, it is important to be in position to disentangle these effects

III

The relationship between litter size and weight of the young expressed by equation (1) or (2) does not undergo any fundamental change during the suckling period

We have followed the growth of a large number of suckling mice by daily weighings up to the end of the 3rd week after birth. The results are given in Table II and in Fig 4. Systematic departures from the straight line relationship seem to occur at birth and shortly afterwards with the small litter sizes, the departures noted in connection with Figs 1 and 2. The birth weights of very small litters seem to be "too low". An explanation for this discrepancy may be that such small litters are artificially brought about by the prenatal death of one or more of the litter. Such prenatal death may easily escape the recorder, who consequently fails to exclude such litters from his statistics. In our new experiments we have taken special care to exclude such cases (*cf* Fig 1) and the fit is markedly improved.

Another systematic departure occurs with very large litters from the beginning of the 2nd week, all the weights are "too low". These irregularities, as well as those mentioned before, are theoretically important.

We have expressed the opinion that there is an equipartition of (for each litter size) a limited supply of nutritive material during intrauterine growth. We have also considered that the ability of the mother to provide nourishment (or, the "drawing power" of the

developing litter) does not increase rectilinearly with the number or the mass of young at birth, but in such a way that if F = the total nutritive supply at any value of N , $\Delta F/F$ is constant for $\Delta N = 1$. Then

$$\Delta F/F = K_2(\Delta W/W) N,$$

or

$$= -k_2 N(\Delta w/w)$$

TABLE II

Number in litter (N), average weight of an individual at birth (w) (from data in Table I), average weight of mother after birth, and estimated increase of weight of mother due to bearing the litter (see text), the estimate of increase of weight in mothers is based upon departures from the known mean growth curve for unmated ♀♀ of this strain, and involves an element of uncertainty as regards the precise age of the mothers

No. in Litter N	Average weight of new born, w	Average weight of mothers, after birth	Estimated increase of weight of mothers
	<i>gm</i>	<i>gm</i>	<i>gm</i>
1	1 63	28 2	6 5
2	1 53	30 5	8 8
3	1 49	28 0	6 3
4	1 41	28 1	6 4
5	1 35	27 4	5 7
6	1 33	27 7	6 0
7	1 31	29 5	7 8
8	1 26	30 0	8 3
9	1 23	30 1	8 4
10	1 20	30 3	8 6
11	1 18	26 7	5 0
12	1 20	31 3	9 6
13	1 14	36 1	14 4

This means that the relative decrease in mean weight of one individual, at birth, will be greater, in inverse proportion to N , for each additional individual in the litter, as result of a proportionate reduction in the nourishment available for each individual. The presence of embryos in the uterus modifies quantitatively the metabolism of the mother. This may be due to the passage of substances from fetus to mother. In any case, the increase in level of nutriment would be expected to be shared between mother and young, on the

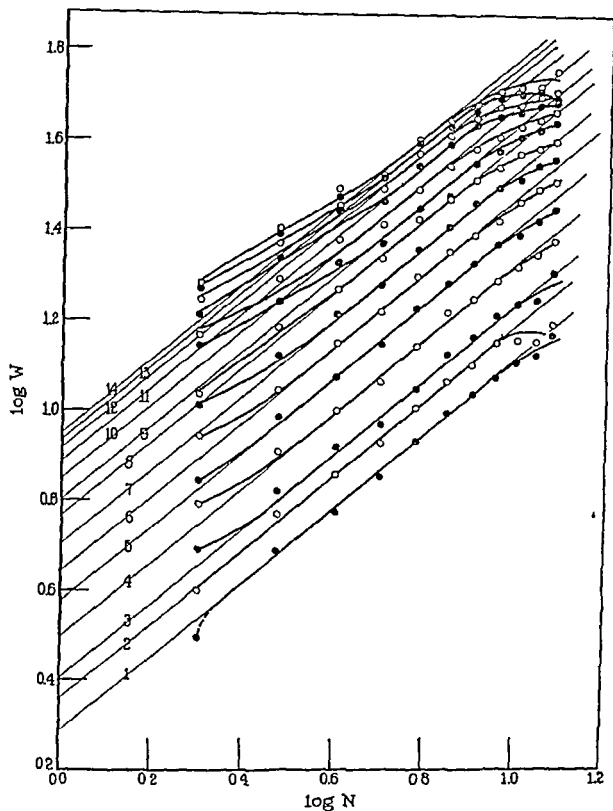


FIG 4 The growth of suckling mice as a function of the size of the litter (Table III), see text

general theoretical basis we are considering. We find this effect in the fact that pregnancy in mice results in a permanent increase of weight of the mother (Table II), the increase is a function of the size of the litter carried, but more comprehensive data are required to examine the point before the nature of the function can be determined.

An increase in F with increasing N could not go on indefinitely, but must reach a limiting value. The approach to a limiting value of the nourishing capacity of the mouse mother is shown in two ways

TABLE III

Average weights of individuals in litters of different sizes, as a function of the number in the litter, at various ages after birth, each litter nursed by one mother (see Fig. 4)

No in litter	No of litters	Average weight of 1 individual ($= W/N$), gm													
		Age, days after birth													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
2	2	1 57	2 00	2 48	3 11	3 51	4 41	5 15	5 45	6 95	7 42	8 19	8 83	9 31	9 62
3	2	1 63	1 97	2 22	2 71	3 21	3 72	4 43	5 12	5 82	6 55	7 30	7 85	8 22	8 49
4	6	1 49	1 81	2 09	2 52	3 99	3 56	4 13	4 68	5 55	6 03	7 00	7 16	7 48	7 80
5	8	1 49	1 78	2 01	2 45	2 93	3 56	4 06	4 45	5 15	5 65	6 31	6 70	7 00	7 31
6	7	1 44	1 71	1 88	2 34	2 83	3 34	3 82	4 38	4 73	5 21	5 86	6 25	6 61	6 70
7	10	1 40	1 68	1 92	2 37	2 76	3 26	3 70	4 25	4 33	5 03	5 60	5 86	6 11	6 34
8	3	1 37	1 60	1 84	2 25	2 68	3 10	3 57	4 10	4 47	4 81	5 43	5 63	5 82	6 03
9	4	1 33	1 59	1 82	2 19	2 62	2 96	3 48	3 89	4 32	4 55	5 04	5 30	5 52	5 73
10	3	1 26	1 45	1 74	2 11	2 49	2 94	3 30	3 76	4 08	4 32	4 63	4 81	5 09	5 27
11	3	1 22	1 31	1 70	2 04	2 42	2 85	3 21	3 52	3 85	4 06	4 38	4 57	4 70	5 05
12	2	1 24	1 31	1 71	2 01	2 36	2 72	3 05	3 34	3 67	3 90	4 15	4 31	4 06	4 80

In all the suckling litters there is a decline of growth rate beginning with the 2nd week of life. This decline is due in part at least to the decline in the milk-secreting capacity of the mother (*cf* Enzmann, 1933). With very large litters the decline is so marked that equation (2) will no longer fit. To keep up the initial growth rate of her offspring a mother suckling 10 or more young would have to produce almost her own body weight in milk every day. Such an increase seems to be beyond the capacity of the mother. Usually the mother kills off part of her litter on reaching the limits of her capacity to

give milk. Our data (Table III) contain only litters which have not been reduced in size by the mother. It is obvious that the large litters are in a state of partial starvation.

MacDowell proved that the growth rate depends on the litter size by artificially reducing litters and by using foster mothers. The litters which were so reduced in size assumed the growth rate characteristic for the new litter size. Still another proof is given by our observation that the *adult* weights are practically the same no matter whether the young were reared in a small or in a large litter. As soon as the suckling period is over and there is an adequate supply of food, the growth rate of mice from large litters increases as compared with the growth rate of small litters, until the two coincide in weight.

If the conditions under which the relationship $\Delta W/W = K (\Delta N/N)$ was deduced should persist unaltered during the suckling period, then the equation should continue to describe the data at equivalent stages of development and K should be fixed and independent of age, K in Fig 4 = 0.84. The essential conditions are (1) that the animals in each litter should remain healthy and should have equal significance as drawers of milk, and (2) that for each additional one in the litter the mother should provide a constant fractional increment of milk (of constant food quality on successive days, in terms of its efficiency in promoting growth of the young—growth being defined as increase in weight). The second condition is interfered with by the time course of milk production by the mother, which seems to be, basically, a function of a cycle of events in the mother released or initiated by parturition,—although the precise form of this cycle is open to modification according to the number of young suckled (Enzmann, 1933). With a small number suckling, the time curve of milk production rises to a flat maximum in the neighborhood of 10 days, with a large number, the maximum comes earlier and the curve is flatter, thereafter it declines (*cf* Enzmann, 1933). In case of suckling mice the intervention of a hypothetical substance supposed to be responsible for "supererogation" of the maternal organism with increasing litter size is unnecessary, it is hard to believe that in the act of suckling substances of such a nature should pass from the infant to the mother. In this case the observation that a more complete

emptying of the mammary gland calls forth a more abundant secretion offers a satisfactory explanation. It is possible that in the case of intrauterine growth a similar explanation might hold, and that hormonal control of the litter weight relation to litter size may be excluded.

Data in Table III, plotted in Fig 4, describe the growth of litters of various numbers, each litter suckled by one mother, as a function of time. For several days after birth larger litters do not receive a

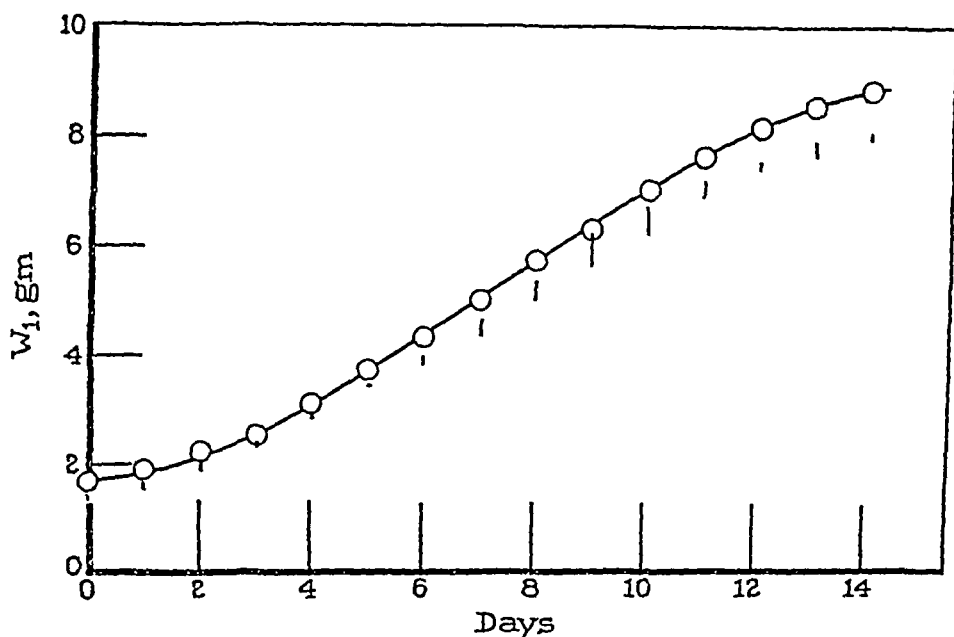


FIG 5 Growth of young suckling mice, w_1 = the ideal weight of a litter of 1, as deduced from the curves in Fig 4, see text. The vertical bars extend between direct determinations made of the average weights of litters of 4, the upper limit of each bar being for females, the lower for males.

supply of milk adequate to maintain $\Delta W/W = K (\Delta N/N)$. Later, at 4, 5, 6 days, the departure of the weights of the very large litters from the line defining K is less, as the peak of the time curve for production of milk is passed, the departure becomes more and more extensive and is apparent with progressively smaller and smaller litters (Fig 4). We have already noted that quite small litters ($N = 1$ or 2) appear at birth "too light," due to death of 1 or more intra-

uterine young, by the 2nd day after birth (Fig 4) this discrepancy is made up and thereafter, as the milk production of the mother increases to a maximum at 8-9 days the young in small litters gain at a more rapid rate, thereafter (9th to 14th day inclusive) the departure is *relatively* less and less, although affecting progressively larger litters. Only in the case of litters containing 5, 6, or 7 young does the relationship $\Delta W/W = K (\Delta N/N)$ remain intact. It is to be understood that in the absence of series of weights upon successive days (as in Fig 4), permitting observation of the gradual change of the curve connecting W with N , one would not be permitted to give much importance to the straight portions of the uppermost graphs in Fig 4. In fact, however, even the slight drop in relative departure of the weights of the smaller litters at the end of the suckling period (12, 13, 14 days) seems quite as expected, since with quite small litters the curve of milk production by the mother falls more rapidly to a low level than is true with larger litters. (The curves drawn for these days, in Fig 4, give only the drift of the plotted points for the smaller litters.) This interpretation, moreover, leads to a kind of consistency in the treatment of the curve of growth during the suckling period. By the method already used in Fig 1, the line for constant K at each day after birth (Fig 4) is projected back to $N = 1$ ($\log N = 0$). One thus obtains a measure of the weight (w_1) of an ideal litter of 1 under the condition that $\Delta F/F = \text{const}$ over a day, the change in w_1 will therefore reflect changes in the growth promoting capacity of the mother, free from the specific interrelation between number in litter and curve of milk yield as a function of time. These magnitudes are plotted in Fig 5. They follow a curve similar to that gotten by direct weighing of suckling young, but higher than the averages so obtained (Fig 5). After the period of maximum milk yield, the rate of growth estimated in this way declines. The curve in Fig 5 thus exhibits the ideal growth curve, free from most of the complications due to varying size of litter as these influence the process of milk secretion, the remaining restriction being that the curve is limited by the condition that the mother supplies a constant fractional increase of nourishment for each addition to the litter. The shape of the curve thus reflects the time course of the capacity of the mother to produce milk.

IV

SUMMARY AND CONCLUSIONS

We have been concerned with the connection between size of litter and weight of litter at birth, especially in mice. The weight at birth represents, it is to be presumed (at least in mice, and for certain other cases), the weight at a particular developmental stage. The connection between number in litter (N) and weight of litter (W) has been interpreted as due to the partition of nourishment between mother and young, and on an equal basis among the several embryos of a litter. The "heterogonic" relationship which the data exhibit between N and W shows that the constant K , defined by

$$\log W = K \log N + \text{const.},$$

is independent of the species, and has an essentially constant value ($0.85 \pm$) in all multiparous mammals, it is therefore regarded as a partition coefficient. In the case of power function relationships between masses of components of a single individual, the respective "drawing powers" of the several organs are diverse, and diverse magnitudes of K are encountered. With developing embryos, the intrinsic drawing powers of the tissues concerned in embryos and mothers are in each case of the same general character, at least among mammals, the constancy of K reflects this. A parallel for the case as it appears in the consideration of relative growth rates of organs in a single individual, and in which the varying magnitudes of the heterogonic growth constant K are presumed to reflect diverse drawing powers of the respective tissues, would be given by intrauterine growth of a litter containing individuals with diverse capacities for growth,—that is, individuals differing genetically with respect to the factors determining the magnitudes of w_1 . We have been dealing with the growth of litters in inbred strains. It is to be presumed that in the case of the growth of a litter containing two categories of individuals so far as concerns intrinsic drawing powers with respect to the nourishment provided by the mother, it would be possible to investigate the way in which K is open to modification. Although difficult, from the standpoint of classifying the individual young, it would appear to be distinctly worth while to make such an experiment, and we have planned it for the future.

It is pointed out that for genetic purposes the ideal weight of a litter of 1 is obtainable from a series of measurements of N and W , free from disturbances affecting the apparent value of this quantity as observed in single births. This weight of an ideal litter of 1 should be employed to disentangle the effects of heterosis and fertility factors from those having to do with individual weight at birth.

During the suckling period the relation $\Delta W/W = K (\Delta N/N)$ is maintained for young mice, but with modifications in the case of small and large suckling litters due to (1) the time course of milk yield, and (2) the effect of litter size upon this. It is shown that a growth curve can be obtained for an ideal litter of 1, under the condition of milk supply that on each day the mother is able to provide a constant fractional increase of milk for each additional young mouse in the litter. The rate of growth then adheres to the time curve of capacity for production of milk.

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THE ALLEGED EFFECT OF ELECTRICAL STIMULATION ON THE METABOLISM OF RED CELL SUSPENSIONS

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INTRODUCTION

In view of a distinction which has been drawn between the metabolic response of a tissue exhibiting excitation ("excitation metabolic effect") and that which results from stimulation not followed by excitation ("stimulation metabolic effect"), Hattingberg (1934*a, b*) has recently carried out experiments on the electrical stimulation of red cell suspensions, which presumably cannot be "excited" in the usual sense of the word. He describes effects on the metabolism of these suspensions which he regards as manifestations of a stimulation metabolic effect pure and simple. His observations can be briefly summarized as follows:

Mammalian and nucleated red cells, suspended in NaCl or phosphate buffers, were placed in Barcroft manometers and stimulated by the passage of a current across two bright platinum electrodes situated on the two sides of the vessel. The stimulation was supplied either by an induction coil or by condenser discharges at the rate of about 25 stimuli per second, and was of such an intensity as would provide from $3(10^{-3})$ to $3(10^{-4})$ cal/sec. The stimulation was applied for 20 seconds out of each minute, and readings of the oxygen consumption were made every 15 minutes. In the case of all the types of blood cell used, the result was found to be an increase in the rate of O_2 consumption of from 70 to 170 per cent. The increase in the rate of O_2 consumption was found to be roughly linear with the amount of current, and, by using Warburg respirometers, it was found that the RQ associated with the increased metabolism is in the neighborhood of unity. Further experiments on suspensions of goose cells, laked by freezing and thawing, showed that this stimula-

tion metabolic effect is not dependent on the cells being intact, but similar results could not be obtained on an inanimate model of charcoal with adsorbed oxalic acid. It was concluded that the effect is the sum of two distinct effects, an increased O_2 consumption, and, under suitable circumstances, an increased anaerobic acid formation, and that it may be due either to a permeability effect or to a modification of the respiratory mechanism.

Because of the obvious importance which would attach itself to a system in which a pure stimulation metabolic effect could be observed, we have repeated Hattingberg's experiments by other and more sensitive methods. Effects such as he describes can certainly be obtained, but the explanation of them is wholly different from that which Hattingberg gives.

Methods

The measurements of " O_2 consumption" were made in a specially designed Fenn respirometer consisting of two cups, of volume 25 cc (including side cups), joined by a capillary of which every centimeter has a volume of 4.5 mm^3 . Mounted in each cup are two electrodes of shiny platinum, 20 mm by 8 mm by 0.1 mm, and about 20 mm apart. To each cup is attached a side cup in which lysin, etc., can be placed, and there are the usual receptacles for KOH. In the initial stages of the investigation, the respirometer cups and electrodes were cleaned with water, alcohol, and ether, but later they were soaked overnight in bichromate cleaning fluid. The respirometer was contained in a water bath at 37.5°C , controlled to within 0.01°C , and was rocked to and fro through an arc of 70° at the rate of 100 per minute. Glass beads were put in the cups to increase the agitation of the fluid, but the presence or absence of the beads does not affect the results.

The stimulating circuit first used was an induction coil circuit, as described by Hattingberg, but this was soon replaced by a circuit supplied by the 60 cycle A.C. mains through a transformer, with a resistance and milliammeter in series. This gives effects identical with those given by the induction coil provided the 60 cycle current is adjusted so as to give the same heat effect in the system as that obtained from the induction coil. The resistance of the material in the respirometers (saline, red cell suspensions, etc.) was measured in every experiment.

After allowing 30 minutes for equilibration, the current was applied for a variable time, usually 8 minutes, and observations of the movement of the drop in the capillary were made every minute until a final equilibrium was reached. While the current is being applied, the readings of the position of the drop must be made with the respirometer in motion. The particular respirometer used leaves

nothing to be desired either as regards sensitivity or its properties as a "dead beat" instrument

Other experimental details will be given in their place

A General Description of the Phenomena

The effect of electrical stimulation on the rate of O_2 consumption of a red cell suspension is easily demonstrated, and Fig 1 shows the typical result for a 30 per cent suspension of rabbit erythrocytes in 1 per cent NaCl. The magnitude of the stimulus applied was 3.1 (10^{-3}) amp/cm² and 6.25 volts/cm, and its duration 8 minutes.¹ The effect is relatively enormous, the resting O_2 consumption of the cells being only 22.2 mm³/gm/hr, and the increase in the apparent O_2 consumption being over 200 per cent. It will be seen that just after the application of the stimulus the graph dips below the line for the resting O_2 consumption, this is clearly a heat effect due to the passage of the current, as Hattingberg observes. Thereafter follows the greatly increased O_2 consumption. The values upon which Fig 1 is based are shown in the first four columns of Table I.

The first four columns of Table I are drawn up in the conventional way, the first column gives time intervals, the second the position of the drop, in cm, in the capillary, the third the movement of the drop in the preceding time interval, and the fourth the apparent rate of O_2 consumption (mm³/gm/hr) in each time interval. The conventional method of expressing the results, however, is a very misleading one in this case, because the movement of the drop is affected by factors which have nothing to do with O_2 consumption, one of them, for example, being the heating effect of the current. It is therefore better to plot, as in Fig 2, the actual position of the drop in the capillary at each time interval. The data upon which Fig 2 is based are shown in the fifth column of Table I (Δz), and are obtained from the figures in the second column by repeated subtraction. When the current is applied, the drop moves away from the stimulated ves

¹ This probably represents a greater stimulus than that used by Hattingberg but the effects are also greater. Hattingberg gives his electrical values in terms of cal/sec, but preferable units are the current density (amp/cm²) and the field strength (volts/cm). The frequency used by Hattingberg (25 per sec.) is less than ours (60 cycles per sec.) and a complete study of the phenomena from a physical point of view should include a study of the frequency dependence.

sel (because of heating), and as movement in this direction corresponds to an *increase* in volume, it is regarded as *positive*, the drop

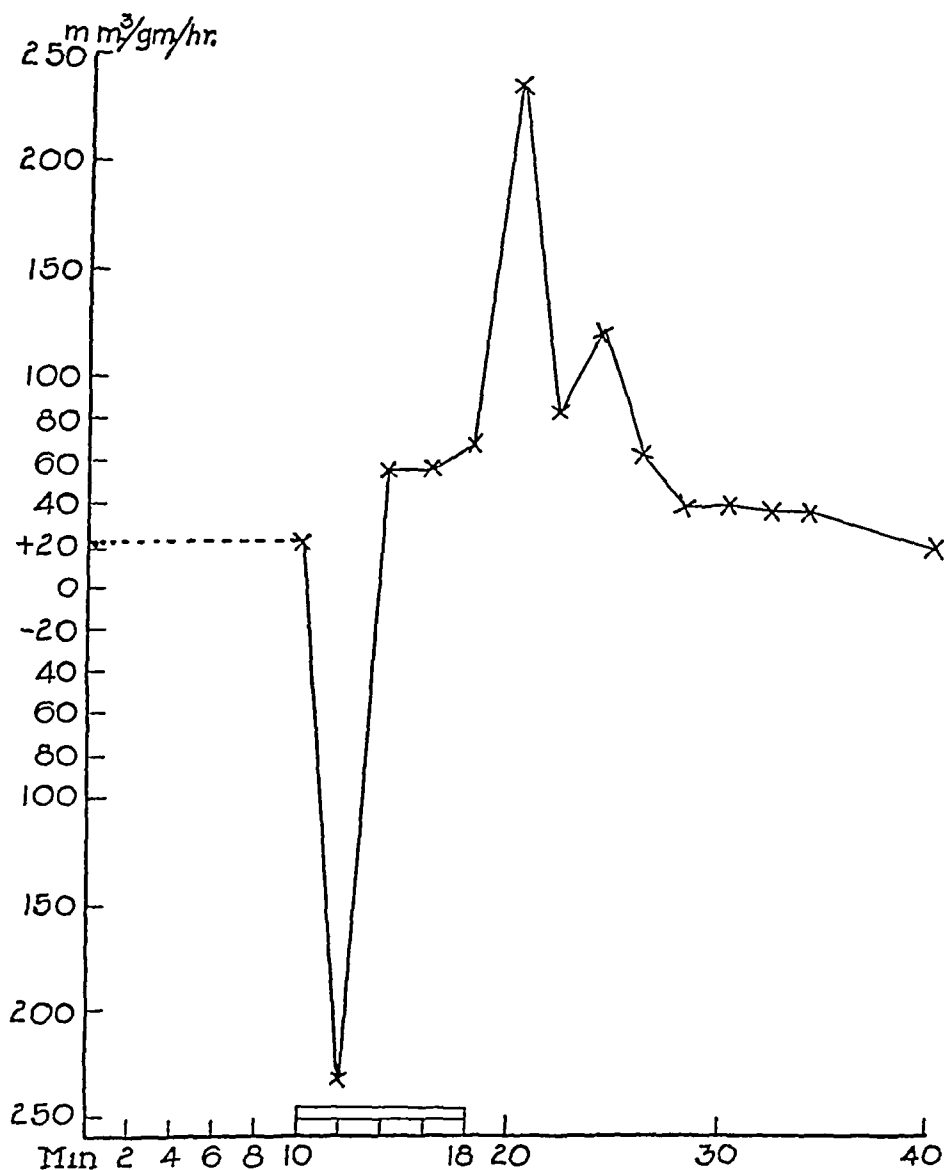


FIG 1 Change in the apparent rate of O_2 consumption of a red cell suspension as a result of electrical stimulation between the tenth and eighteenth minutes Ordinate, O_2 apparently consumed, $mm^3/gm/hr$, abscissa, time in minutes The resting O_2 consumption is $22.2 mm^3/gm/hr$

then returns towards its equilibrium position, which it soon reaches. The current being turned off, however, the drop then proceeds for a considerable distance in the direction of the stimulated vessel, this movement is considered *negative*, since it corresponds to a *decrease* in volume of the stimulated system. Conventionally, it would be regarded as evidence of an O_2 consumption.

In Fig 2, the application of the current is followed by a small heat effect (positive), which is not maintained, and even while the current

TABLE I

Time	Reading of drop	Δ_1	Rate	Δ_2	Δ_3
min			mm ³ /gm/h		
0	7.4	—	—	—	—
10	6.6	0.8	22.2	-0.8	0.0
Current on					
12	8.2	-1.6	234	0.8	1.76
14	7.8	0.4	58	0.4	1.20
16	7.4	0.4	58	0.0	0.64
18	6.95	0.45	69	-0.45	0.03
Current off					
20	5.3	1.65	241	-2.1	-1.46
22	4.7	0.6	86	-2.7	-1.90
24	3.85	0.85	124	-3.55	-2.59
28	3.15	0.70	42	-4.25	-2.89
34	2.35	0.80	42	-5.05	-3.21
40	1.87	0.48	22	-5.50	-3.21

is still applied the drop moves back towards its equilibrium position. When the current is turned off there is an accelerated movement of the drop in the negative direction, this corresponding to the large peak in Fig 1, which, if considered as a rate of O_2 consumption, would correspond to a rate of about 800 per cent greater than the resting rate. The drop continues to move in the negative direction with decreasing rapidity, until it is moving at the rate corresponding to the resting O_2 consumption. Comparing Figs 1 and 2, it will be apparent that the conventional way of representing the results greatly

exaggerates and in some respects falsifies the state of affairs, the heat effect, for example, is magnified in Fig 1 and at the same time is too short, and the peak in the rate of O_2 consumption is partly due to the fact that the current has been turned off and the heat dissipated

In order to eliminate the continuous movement of the drop which results from the resting respiration of the cells, it is even better to use the respirometer differentially, cells being placed in both vessels, and

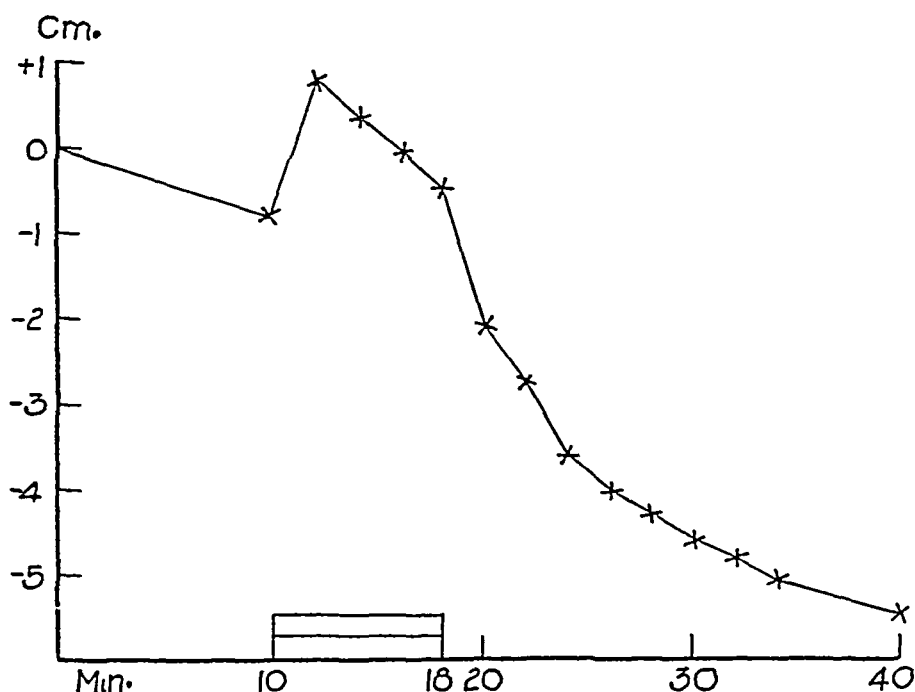


FIG 2 Same results as in Fig 1, but plotted with actual movement of the drop, in cm, on the ordinate

the contents of one being stimulated. Elimination of the effect of the resting metabolism on the movement of the drop leads to the figures in the sixth (Δ_3) column of Table I, from which Fig 3 is plotted. It is similar to Fig 2, except that the heat effect appears somewhat greater and that the drop passes to a position of final equilibrium after some 30 to 40 minutes, just as it would do if oxygen were used up as a result of the stimulation.

It did not occur to Hattingberg, apparently, to try the effect of

stimulating a 1 per cent NaCl solution in which no cells were present Fig 4, plotted in the same way as Fig 3, shows what happens when this is done. There is a heat effect which is not maintained, and this is followed by a movement of the drop in the negative direction, *i.e.* past its original equilibrium point, to an extent which would correspond to an apparent O_2 consumption of about 13 mm^3 . This effect we shall call the "NaCl effect"². It will be clear that when red cells are stimulated in a medium consisting of 1 per cent NaCl, the NaCl

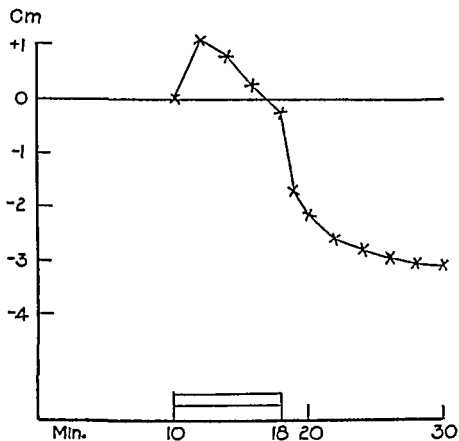


FIG 3 Same results as in Fig 2 plotted in the same way, but with the resting O_2 consumption eliminated by the use of a differential respirometer

effect must add itself to any stimulation metabolic effect attributed to the presence of the cells. It is true that the apparent O_2 consumption observed when a suspension of red cells is stimulated is always greater than that obtained by stimulating either 1 per cent NaCl or

² It is to be emphasized that the new equilibrium which corresponds to a diminution in the volume of the stimulated system is a permanent one and that the drop remains at the new equilibrium position for at least 6 hours. This applies to all the volume decreases described in this paper.

the supernatant fluid in which the cells are suspended, and this might lead one to think that there is a stimulation metabolic effect, attributable to the presence of the cells, and in excess of the NaCl effect. Unfortunately, all the effects (except that due to heat) disappear when the electrodes are platinized, and it will be shown below that what has been considered as an O_2 consumption is really an electrode artifact.

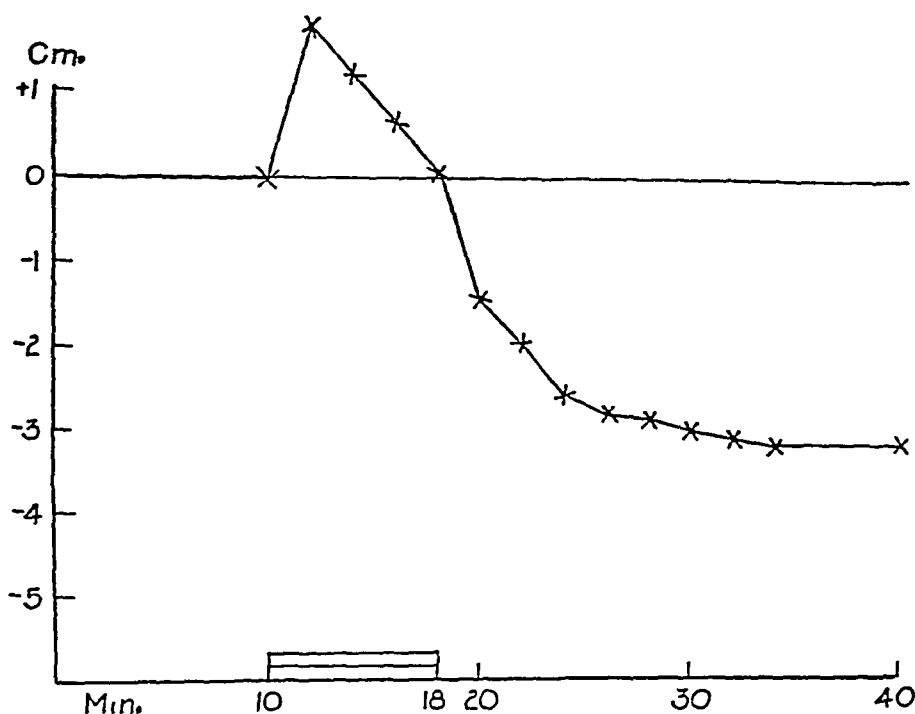


FIG 4 Effect of the passage of current through a 1 per cent NaCl solution. Ordinate, movement of drop, in cm. of capillary, abscissa, time in minutes. The current is applied between the tenth and eighteenth minutes.

The NaCl Effect

That the NaCl effect, i.e. the diminution in volume obtained when a current is passed between shiny platinum electrodes through a 1 per cent NaCl solution, is an electrode artifact is shown by the fact that it disappears when the electrodes are platinized. A typical experiment is shown in Table II and Fig 5.

These results are plotted in Fig 5. It will be seen that the NaCl

effect completely disappears when the electrodes are platinized, and that the increase in volume due to heating is maintained during the entire period of stimulation, while when the electrodes are shiny the initial increase in volume is not maintained, but is followed by a volume decrease even while the current is still on. It is obvious that in the case of the shiny electrodes the volume increase due to heat is being off set by a volume decrease (the NaCl effect), which apparently begins as soon as the current is applied. For this reason the size of

TABLE II

Time <i>min</i>	Movement of drop <i>cm</i>	
	Shiny electrodes	Platinized electrodes
0	0 00	0 00
2	1 10	1 45
4	0 80	1 60
6	0 25	1 65
8	-0 25	1 70
	Current off	Current off
9	-1 70	0 50
10	-2 15	0 30
12	-2 60	0 10
14	-2 80	0 05
16	-2 95	0 00
18	-3 05	0 00
20	-3 10	0 00

the heat effect in these systems does not depend on the square of the current, but rather on the current itself.

The NaCl effect obtained with shiny electrodes seems to occur equally well irrespective of the nature of the gas phase above the liquid, as can be shown by evacuating the system and replacing the atmospheric gases with nitrogen, hydrogen, or helium. It is, of course, very difficult to be sure that the replacement is complete, but we are certain that it is complete to the extent of 99 per cent at least, and that the magnitude of the volume decrease which follows stimulation is not appreciably altered. As might be expected, it is immaterial whether or not KOH is placed in the respirometer cups.

In view of the fact that the gas phase does not seem to be directly

involved, we tried to obtain the effect with substances other than NaCl. All the substances were made up so as to be equivalent, as regards conductivity, with a 1 per cent NaCl. Table III shows some typical results, and the magnitude of the effect can be judged from the decrease in volume observed at final equilibrium. In all cases the stimulus was $4.7 (10^{-3})$ amp/cm² and 6.25 volts/cm applied for 8 minutes.

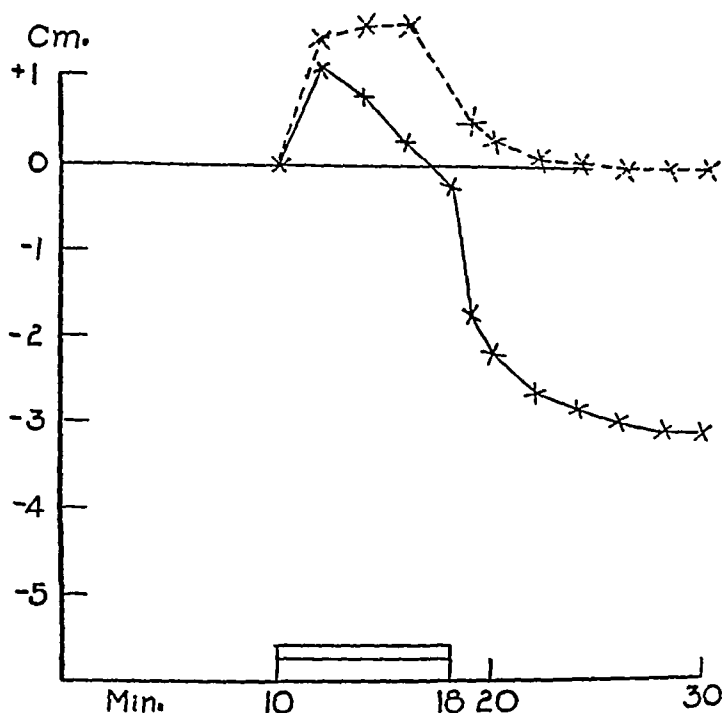


FIG 5 Similar to Fig 4 full curve, shiny electrodes, broken curve, platinized electrodes

The volume decrease occurs with all the halogen salts, and its magnitude is roughly proportional to the ease with which the halogen radicals are displaced. There is a somewhat smaller effect with the carbonates, but none with nitrates, phosphates, or sulfates.

In the case of the halogen salts the volume decrease is accompanied by the production of I_2 , Br_2 , or Cl_2 , as the case may be, the greater part of the gases apparently being in solution. Since the gases were found to appear either in very small quantities or not at all when the

electrodes were platinized (under which condition the volume decrease is also absent), we thought that there might be a direct relation between the amount of titratable I_2 , Br_2 , and Cl_2 , and the magnitude of the volume decrease. The relation, if any, appears to be a very imperfect one, for although the average amount of titratable I_2 (0.46 millimols/litre) and Br_2 (0.63 millimols/litre) is consistently greater than the average amount of titratable Cl_2 (0.28 millimols/litre), the correlation coefficient between the volume decrease and the amount of titratable halogen is only about 0.2. Very little reliance, however, can be placed on these quantitative estimations, for the results are

TABLE III

Substance	Volume decrease
	mm
KI	18.0-29.7
KBr	21.1-28.1
NaCl	13.9-18.0
LiCl	14.9
K_2CO_3	12.3
KCl	9.5
$MgCl_2$	5.9
NaF	1.2
KNO_3	0.0
KH_2PO_4	0.0
K_2SO_4	0.0
$MgSO_4$	0.0

greatly dependent on the state of the electrodes, and this seems to vary from time to time.

Early in the course of the investigation we noticed that the NaCl effect and the apparent O_2 consumption observed when red cell suspensions are stimulated both tended to become less when the stimulation was repeated. At this time the respirometers and the electrodes were being cleaned after each experiment with water, alcohol, and ether. Shortly afterwards we noticed that the effects were becoming very much smaller on successive days, and were, in fact, tending to disappear altogether. Suspecting that the electrodes had become "dirty," we cleaned the entire apparatus with a bichromate

cleaning solution, and this resulted in the re-appearance of the effects, on a greater scale, indeed, than ever before. Since then we have found that the ease with which the electrodes are "poisoned" or "saturated" depends on the amount of volume decrease. For example, in a solution of KI the volume decrease produced by the passage of 75 milliamps for 8 minutes is about 30 mm³ when the electrodes are freshly cleaned, a second passage of the same current, however, may produce only about half of this effect, and succeeding stimulations will produce even less. It is necessary to soak the electrodes for more than 24 hours in cleaning fluid in order to restore them to their original condition. In a solution of NaCl, on the other hand, this poisoning of the electrodes is much less apparent, just as the volume decrease obtained is smaller. The electrodes, moreover, are much more readily "cleaned."

While these effects on volume clearly come under the category of electrode phenomena, it is extremely difficult to imagine the type of reaction which would result in a permanent decrease in volume. The effects seem to be associated with electrode reactions in which Cl₂, Br₂, and I₂, among other things, are formed, and these particular reactions seem to occur only at shiny platinum electrodes, at which the potential drop is great. One is inclined to suggest that the volume decreases may be due to the adsorption of some substance on the electrodes, in which case the poisoning referred to above may be due to the electrodes becoming saturated with this substance. It must be admitted, however, that this suggestion is quite speculative.³

³ It is perhaps significant that the volume decrease continues to develop for several minutes after the current has been turned off. This suggests that a new equilibrium is being attained comparatively slowly, limited, perhaps, by diffusion rates. Although the little evidence we have is against the gas phase being directly involved in a chemical sense, it is quite possible that the primary effect takes place in the liquid phase and in the neighborhood of the electrodes, and that a new equilibrium is then reached between the gas phase and the liquid phase, so that the decrease in volume observed is really a decrease in volume of the gas phase, occurring irrespective of the nature of the gas and consequent upon chemical changes in the fluid. The matter might be further investigated by means of dilatometer experiments.

Effects Observed on Stimulating Red Cell Suspensions

The diminution in volume observed when red cell suspensions in 1 per cent NaCl are stimulated by the passage of a current is always greater than that obtained by the passage of the same current through

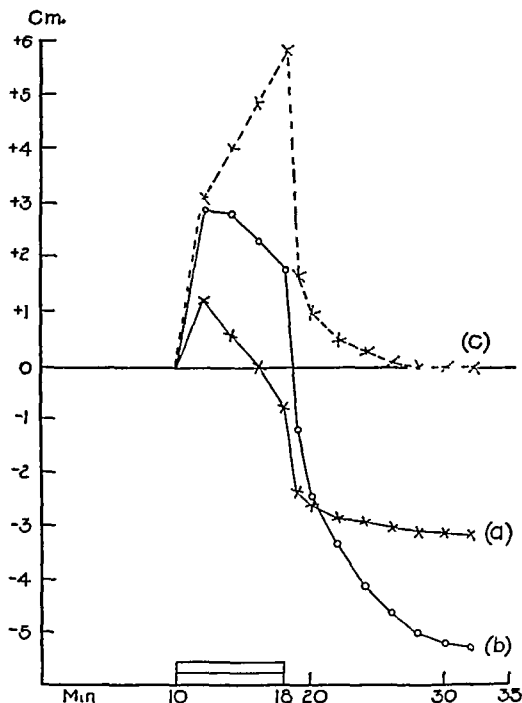


FIG 6 Passage of current (a) through 1 per cent NaCl with shiny electrodes, (b) through red cell suspension with shiny electrodes, and (c) through red cell suspension with platinized electrodes Ordinate and abscissa as in Fig 4

1 per cent NaCl, but this effect is also an electrode artifact, which disappears completely on platinizing the electrodes. Fig 6 shows the results obtained by stimulating (a) 1 per cent NaCl with shiny electrodes, (b) a 30 per cent suspension of rabbit red cells, also with shiny electrodes, and (c) the same cell suspension with platinized

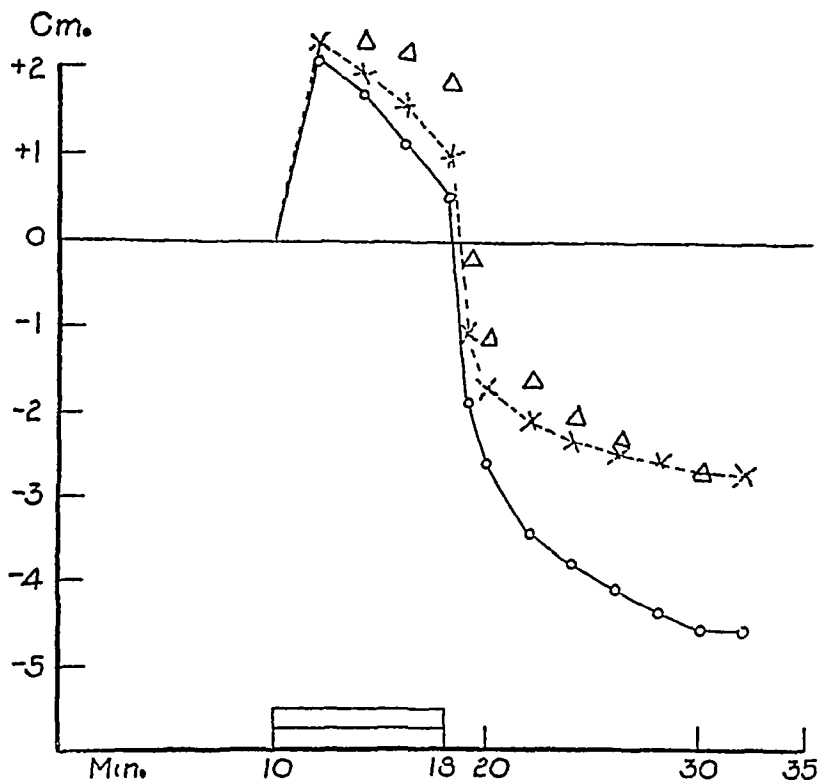


FIG 7 Passage of current through red cell suspension (circles), suspension of stromata (crosses), and hemolyzed cell contents (triangles) Ordinate and abscissa as in Fig 4

electrodes. In each case the stimulus applied was $4.7 (10^{-3})$ amp / cm^2 , and 6.26 volts/cm for 8 minutes.

It is this diminution in volume which Hattinberg has apparently mistaken for an increased O_2 consumption resulting from stimulation. From the point of view of cell metabolism, etc., it is no doubt academic to discuss the properties of an electrode artifact, but there is evidence that the effect observed in the case of red cell suspensions is not

merely the NaCl effect on a large scale (a) The effect obtained in the case of suspensions of intact red cells is greater than that found in the case of either stroma suspensions or cell contents obtained by freezing and thawing, even when the resistance of the contents of the respirometer is kept constant by adjusting concentrations (see Fig 7) The magnitude of the effect therefore appears to depend on the cells being intact (b) It does not depend, however, on the cells being "alive" for as great effects are obtained with suspensions of red cells treated with 0.6 per cent formol as with suspensions of normal cells Formol treated cells show no resting oxygen consumption (c) While it is certain that the NaCl effect is not modified by the presence or absence of KOH in the respirometer cups, it is by no means certain that the effects observed in the case of red cell suspensions are not modified In some experiments we have found a smaller volume decrease when KOH is absent in others, there has been no appreciable difference

If this last point could be decided, it would enable us to say more definitely whether the effects observed in the case of suspensions are simply the NaCl effect on a large scale, or the NaCl effect together with an additional effect of a different kind, and the point has an obvious bearing on the respiratory quotients which Hattinberg has reported as associated with red cell suspensions which have been stimulated (R.Q.'s of 0.8, increasing to about 1.0) The point, however, is almost impossible to decide, for if we attempt to decide it we have first to pass the current through the suspension with KOH present, and then to pass it with KOH absent (or *vice versa*) If we do not clean the electrodes thoroughly between the two stimulations, we find a smaller volume decrease on the second stimulation, this is clearly due to the contamination of the electrode as a result of the first stimulation, but the difference might be erroneously put down to presence or absence of the KOH, and equally erroneously regarded as evidence for there being an R.Q. If we try to clean the electrodes between stimulations, this process requiring about 24 hours immersion in cleaning solution, it is clearly impossible to use the same cell suspension for the second stimulation⁴

⁴ It is only fair to Hattinberg to point out that he could not possibly have observed the final decreases in volume, because he apparently worked with non

Effects Observed on Stimulating Serum

When currents of the same magnitude as above are passed through serum, as opposed to NaCl and red cell suspensions, there appear to be still other electrode artifacts. With shiny platinum electrodes, there is a relatively enormous heat effect which steadily increases as

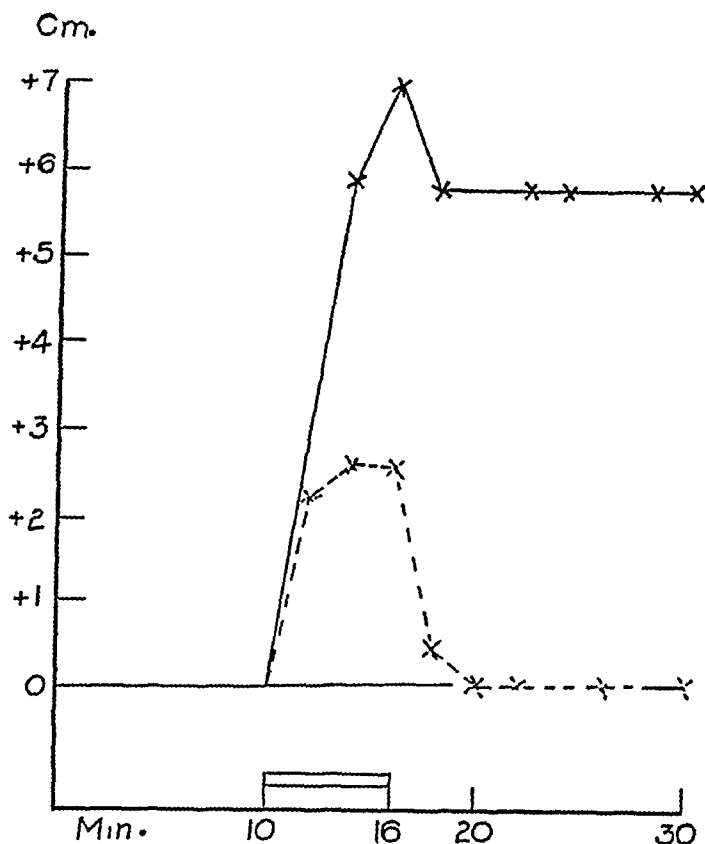


FIG 8 Passage of current through serum. Full curve, shiny electrodes, broken curve, platinized electrodes. Current applied between tenth and sixteenth minutes. Ordinate and abscissa as in Fig 4.

the current is flowing, and this effect is so great that the period of stimulation has to be reduced lest the drop run out of the capillary. When the current is turned off, the drop moves back towards the

differential respirometers and interpreted all changes in terms of "rates of O_2 consumption". What his R Q's mean is impossible to say, for respiratory quotients have no meaning in connection with these systems.

equilibrium position, but soon stops at a new equilibrium position which represents a marked *increase* in volume. This new equilibrium is maintained, apparently, indefinitely. When the electrodes are platinized, the heat effect is much less, and the drop promptly returns to its original equilibrium position when the current is turned off (see Fig. 8).

Like the volume *decreases* observed in the case of NaCl, red cell suspensions, etc., the volume *increase* obtained in the case of serum undergoes a steady diminution in magnitude on successive stimulations. Again, and for the same reasons as given in the case of the cell suspensions, we have been unable to decide whether the presence or absence of KOH modifies the effect.

SUMMARY

The apparent increase in the rate of O_2 consumption described when an alternating current, or induction coil current, is passed through a red cell suspension (in a buffered NaCl solution) is not a metabolic effect in any sense of the word. The passage of the current results in a permanent volume decrease in the system, and it is this which has been erroneously interpreted as an "increase in the rate of O_2 consumption." Its magnitude is about 1 part in 1000. The utilization of O_2 is not involved at all, and the same effect is obtained, on a somewhat smaller scale, when the current is passed through a solution of NaCl or of the other halogen salts. The effects occur only with shiny platinum electrodes, and disappear entirely when the electrodes are platinized. Passage of the current through serum, on the other hand, results in a permanent increase in the volume of the system, this effect also disappearing on platinization of the electrodes. The effects are apparently related to obscure electrode phenomena.

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ACETYLATION OF TYROSINE IN PEPSIN

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In an earlier paper (1) the writer described the preparation and isolation of three crystalline acetyl derivatives of pepsin. They were, "100 per cent active" acetyl pepsin in which the 3 or 4 primary amino groups of pepsin had been acetylated with less than 15 per cent change in the specific activity, "60 per cent active" acetyl pepsin which contained 6-11 acetyl groups per molecule, and "10 per cent active" acetyl pepsin which had 20-30 acetyl groups per molecule of protein. Reversion of the 60 per cent active enzyme into the 100 per cent active was effected by treatment with normal sulfuric acid at 5°C¹. It was pointed out in this earlier work that in all probability the primary amino groups belong to the lysine part of the protein molecule and that since acetylation of these groups failed to produce any appreciable effect on the activity of pepsin that the rôle played by the lysine in the enzymatic activity of the molecule is probably relatively small.

Since the introduction of a few acetyl radicals into other groups of the protein molecule definitely diminished the specific activity, it was reasoned that these groups of the protein must be more closely related to the seat of the enzymatic activity.

The present work was undertaken to determine the structural position of these few acetyl groups which have such a pronounced effect on the activity of pepsin. Though the evidence is somewhat indirect it nevertheless seems probable that those acetyl groups in the 60 per cent active acetyl pepsin which are responsible for the decrease in specific enzymatic activity are attached to the phenolic hydroxyl groups of some of the tyrosine components of the protein.

¹ In the previous paper, *J. Gen. Physiol.* 1934, 18, 53-54, the temperature is given as 10°C. This should read 5°C.

In the 60 per cent active preparation there are three acetyl groups which are not in the 100 per cent active acetyl pepsin. These three additional acetyl groups are rapidly hydrolyzed by molar acid or by alkali at pH 10.0, whereas the acetyl groups on the amino groups are not hydrolyzed under the same conditions. With this property in mind these easily hydrolyzed acetyl groups have been designated as "pH 10.0 labile" acetyl groups. There are three less tyrosine phenol groups in the 60 per cent active acetyl pepsin as measured colorimetrically with the Folin phenol reagent under conditions which will not hydrolyze an acetylated phenol.

When the 60 per cent active material is changed back into 100 per cent active acetyl pepsin by the above mentioned acid treatment there is an accompanying loss of the pH 10.0 labile acetyl groups and the number of tyrosine phenol groups returns to that of the original pepsin.

EXPERIMENTAL RESULTS

Preparation—A slight modification in the method of preparation of the 60 per cent active acetyl pepsin has led to more uniform and reproducible results. Table I contains the analyses of several different acetyl derivatives and of pepsin. In this table the 100 per cent active acetyl pepsin was prepared by acid hydrolysis of the 60 per cent active materials. A discussion of the procedures and the interpretation of the analyses will be found later in this paper but it might be noted at this time that the acetyl group figures are more significant than the tyrosine phenol group figures for the determination of the former is subject to less error and the interpretation of the figures is on a more sound chemical basis.

From Table I it appears that with the change from pepsin to 60 per cent active acetyl pepsin there is an increase of three pH 10.0 labile acetyl groups (besides the increase of 3 or 4 acetyl groups on the primary amino groups). There is also a decrease in the tyrosine-tryptophane value of the protein as measured by the colorimetric "pH 8.0 method," equivalent to 3 tyrosine phenol groups.

Experimental Procedure

In general the materials, the analyses of which appear in Table I, were prepared by the methods described previously (1). In the present instance, however, the

60 per cent active acetyl pepsin was prepared in the same way that was previously described for the preparation of 10 per cent active acetyl pepsin except that the acetylation by ketene was stopped when the specific activity was approximately 60 per cent of the original pepsin. This point was determined by analyzing samples taken from time to time. The material was then precipitated from solution by acidification and half saturation with magnesium sulfate, fractionated, crystallized, and finally dialyzed in collodion bags for 24 hours on a dialyzer (2).

TABLE I
Acetyl and Tyrosine Analyses of Pepsin and Its Acetyl Derivatives

Name	No	[P U] Hb mg P N	Tyrosine-tryptophane content by colorimetric method					No of acetyl groups per mol pepsin	
			Per cent protein		No of groups per mol pepsin			pH 10 labile	Total
			pH 11.0 method	pH 8.0 method	pH 11.0 method	pH 8.0 method	(pH 11- pH 8)		
3 x cryst. P D pepsin	1	0 21	11 8	11 8	24	24	0	0 0	1
5 x " " "		0 20	11 7	11 7	24	24	0	0 0	
100 per cent active acetyl pepsin†	20	0 17	12 0	11 5	24	23	1	0 2	
" " " †	24	0 18	11 7	12 0	24	24	0		
" " " †	11	0 18						0 3	3
60 per cent active acetyl pepsin†	11	0 12	11 8	10 4	24	21	3	3 6	7
" " " †	16	0 12	12 0	10 7	24	21	3	3 0 ± 0 2	6
10 per cent active acetyl pepsin‡	6	0 037	12 0	8 2	24	16	8	14	18
" " " §	6	0 036	12 2	7 7	24	15	9	13	16

* See discussion under Experimental methods for the determination and calculation of these figures

† Crystallized and fractionated

‡ Reaction mixture *z e*, not fractionated

§ Fractionated but not crystallized

at 5°C against $m/2000$ pH 4.65 acetate buffer. All preparations and samples were dialyzed as just described before final analysis. The 100 per cent active acetyl pepsin preparations were made by subjecting the 60 per cent active preparations to 1.25 N sulfuric acid at 5°C for 75–100 hours followed by concentration, fractionation, crystallization, and dialysis. The activity estimation was made by the hemoglobin method of Anson and Mirsky (3). The other analyses were carried out as described under Experimental methods.

On treatment of the 60 per cent active acetyl pepsin with normal sulfuric acid at 5°C the specific enzymatic activity of the protein

risers to that of pepsin.² The pH 10.0 labile acetyl groups are no longer detectable and the tyrosine-tryptophane value has increased to that of pepsin. It will be shown later that it is highly probable that these changes in tyrosine-tryptophane value are due to the coupling or hydrolysis of acetyl groups on tyrosine phenol groups of the protein. It is concluded, therefore, that the change in specific activity from 100 per cent active acetyl pepsin to 60 per cent active acetyl pepsin can be attributed to the acetylation of 3 tyrosine phenol groups in the protein. It is possible that not all 3 of these tyrosine phenol groups are involved in the effect produced on the activity but the writer has endeavored without success to obtain decisive evidence on this point.

In the change from 60 per cent active acetyl pepsin to the 10 per cent active acetyl pepsin there is a further increase in pH 10.0 labile acetyl groups and a decrease in tyrosine-tryptophane value of the protein. The change in the number of acetyl groups, however, is not equivalent to the decrease in number of tyrosine phenol groups calculated from the chromogenic value so that some of these acetyl groups may be attached to other than tyrosine phenol groups.

Tyrosine Content of Pepsin

It was pointed out by Wu (4) that the color produced by the phenol reagent in the presence of proteins is largely due to the tyrosine in the protein. Since two other amino acids, tryptophane (5) and cysteine (6) produce the characteristic blue color with the phenol reagent they must be considered as possible sources of color when a protein is treated with alkali and the phenol reagent. There is also the possibility that there exists in some proteins a component other than the amino acids and that this component will reduce the phenol reagent. Heme of hemoglobin is such a component and is known to reduce the reagent (6). Amino acids which in the pure state do not produce the color with the reagents may, when in combination with

² The figures given for the specific activity of the 100 per cent active acetyl pepsin are some 10-20 per cent below that of pepsin. Preparations have been obtained with a specific activity more nearly that of pepsin ($[P-U]^{Hb} \text{ mg P.N.} = 0.20-0.22$) but a small fraction of these preparations seems to be unstable with respect to activity and is lost on standing, the result being a lowering in the specific activity of the total material.

other amino acids in the protein, then have the property of reducing the color reagents. There is no evidence for these possibilities in the case of pepsin except for one observation,³ and there seems to be no correlation of this with the present work. Pepsin gives a negative nitro prusside test for free S H groups. Cysteine is, therefore, probably not present and the color giving property of pepsin is due to the tyrosine and tryptophane.

The estimation of the tyrosine tryptophane content of pepsin was carried out in the present instance by two related colorimetric methods in which Folin's phenol reagent (7) was used. The conditions of one method, designated as the pH 8.0 method, are so arranged that free tyrosine phenol groups may be determined in the presence of, but without the hydrolysis of, acetylated phenol groups. The conditions of the other method, designated as the "pH 11.0 method," are such as to hydrolyze the acetylated phenols and then measure the total number of phenol groups with the same reagents and under the same conditions as the pH 8.0 method employs. From these two methods the total (free plus acetylated) phenol groups and the free phenol groups are determined. The difference between the two designates the number of acetylated groups. A discussion and an outline of the procedures are to be found in the section devoted to Experimental methods.

Rate of Hydrolysis of pH 10.0 Labile Acetyl Groups and Diacetyl Tyrosine in Acid

It was brought out in our earlier work (1) that there is a difference in some of the acetyl groups of 60 per cent active acetylated pepsin with respect to acid hydrolysis. Those which are attached to the primary amino groups are hydrolyzed by acid with comparative diffi-

³ When to solutions of pepsin or other proteins of such a concentration as is used in measuring the chromogenic value by phenol reagents is added 1 ml. of 0.002-0.0005 M CuSO_4 the chromogenic value is increased from 1-3 times. This increase in color value is not demonstrable with acid hydrolysates of pepsin though it is with enzymatic hydrolysates. The increase is very noticeable on purified gelatin which according to the accepted analyses, contains little or no tyrosine and tryptophane. Proline or a pyrrolle type component is suspected though in the pure state and in the presence of CuSO_4 proline and hydroxy proline show no action toward the phenol reagents.

culty Fig 1 shows graphically the results of an experiment in which a solution of diacetyl tyrosine, prepared as directed by Bergmann and Stern (8), and a solution of 60 per cent active acetyl pepsin were hydrolyzed by 1.25 N sulfuric acid at 5°C. Experimental difficulties prevented measurement of the tyrosine-tryptophane color value and the acetyl estimation during the hydrolysis of the enzyme solution. The pH 10.0 labile acetyl analysis was, however, performed on the initial and final products and is included in Fig 1. The experiment

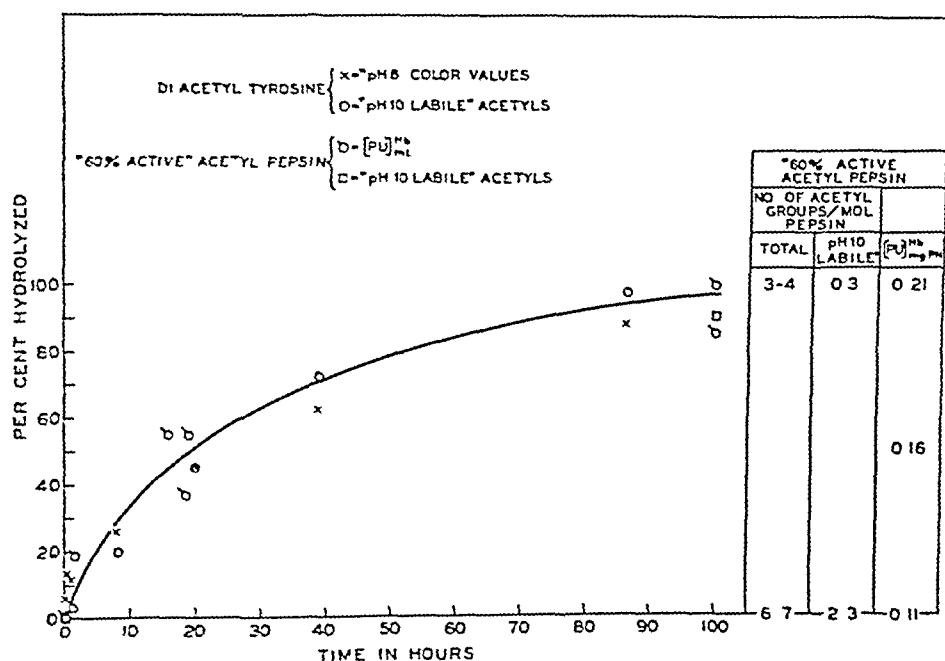


FIG 1 Rate of hydrolysis of 60 per cent active acetyl pepsin and of diacetyl tyrosine in 1.25 N sulfuric acid at 5°C

confirms the experiments of our previous paper in which it was demonstrated that in strong acid the specific activity of the enzyme returns to approximately that of pepsin and the acetylated protein loses some of its acetyl groups. In addition it shows that the rate at which this reactivation takes place is very close to the rate at which the acetyl group on the phenol group of diacetyl tyrosine is hydrolyzed under identical conditions.

Experimental Procedure

Enzyme—250 ml of a dialyzed preparation of 60 per cent active acetyl pepsin containing 3.0 mg P N/ml was cooled to 5°C, added to 250 ml of cooled 2.5 N sulfuric acid, and the suspension stirred continuously. Under these conditions a large part of the protein is insoluble. Samples were taken from time to time, the precipitate filtered off, and the acid filtrate neutralized with an equal volume of 1.5 N sodium acetate. Analyses for protein nitrogen and enzymatic activity were made on this neutralized filtrate. When the specific activity had reached that of pepsin the total protein was precipitated, fractionated, crystallized, and analyzed for specific activity, pH 10.0 labile, and total acetyl groups. A sample of the original 60 per cent active pepsin was analyzed at the same time.

Diacetyl Tyrosine—0.5 gm of crystalline diacetyl tyrosine was dissolved in 79 ml water with the aid of 1 ml of M/1 pH 5.0 citrate buffer. 70 ml of this solution was cooled to 5°C and added with stirring to 70 ml of 2.5 N sulfuric acid at 5°C. 20 ml samples were taken from time to time and analyzed for free acetic acid by distillation from a 3 molar citrate buffer pH 4.0 and subsequent titration of the distillate. Samples were also analyzed for free phenol groups by the pH 8.0 method. In the early part of the hydrolysis the measurement of free phenol groups was made possible by adding known quantities of tyrosine to the aliquot of reaction mixture being analyzed by the pH 8.0 method and then corrected for in the calculation. This reduced the error due to comparison of widely different colorimeter readings.

Rate of Hydrolysis of pH 10.0 Labile Acetyl Groups and of Diacetyl Tyrosine in Alkali

Acetyl groups may be hydrolyzed by alkali as well as by acid and so an experiment was performed at pH 9.0–10.0 to see if the acetyl group on the phenol group of tyrosine is hydrolyzed under the conditions which hydrolyze those on 60 per cent active acetyl pepsin⁴. The results are shown in Fig. 2. Unfortunately the pH of the medium was not exactly the same for the two materials, that of the protein solution being pH 9.8, compared to pH 9.0 of the diacetyl tyrosine solution (pH measurements by hydrogen electrode). Since the enzyme is immediately inactivated under these conditions the activity

⁴ In the medium for hydrolysis pH 9.0–11.0, of the labile acetyl groups a glycine buffer and probably any material containing free amino groups should be avoided in high concentrations (0.2 molar or greater) for it was found with glycine that the amino group acts as an acceptor of the liberated acetyl group after hydrolysis. It is, therefore, not free to be estimated as acetic acid and is hydrolyzed from glycine only after relatively vigorous hydrolytic treatment.

could not be followed. The change in the tyrosine-tryptophane value of the protein by the pH 8.0 method was followed along with the change in acetyl groups. It seems perfectly clear from Fig. 2 that at pH 9.0–10.0 the hydrolysis of acetyl groups from 60 per cent active acetyl pepsin is accompanied by a corresponding increase in the pH 8.0 chromogenic value of the protein. Also that the hydrolysis of the oxygen acetyl linkage of diacetyl tyrosine takes place at approximately

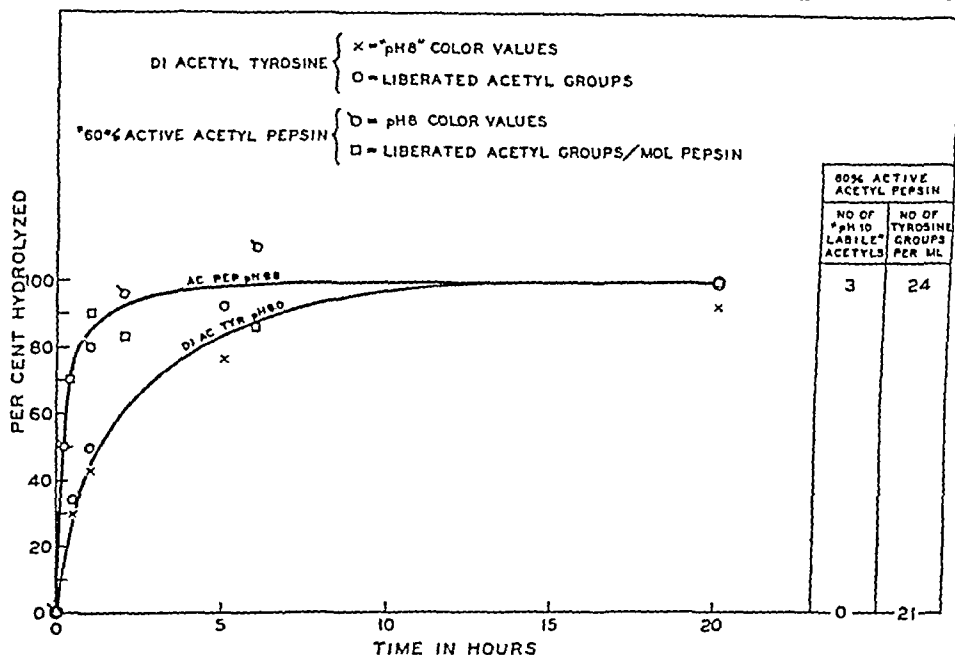


FIG. 2 Rate of hydrolysis of 60 per cent active acetyl pepsin and diacetyl tyrosine at pH 9.0–10.0 and 35°C

the same rate as does the hydrolysis of the pH 10.0 labile acetyl groups of the above mentioned 60 per cent active acetyl pepsin

Experimental Procedure

Enzyme—To 41 ml of 60 per cent active acetyl pepsin containing 13 mg P N/ml was added with stirring a solution of 5.2 ml 1 N sodium hydroxide and 23.8 ml M/10 borate buffer pH 10.5, final pH 9.8 determined by the hydrogen electrode. The solution was kept at 35.5°C throughout the experiment. Samples were taken at varying intervals of time and analyzed for free acetic acid by distillation from 3 M citric acid with subsequent titration of the distillate. Analyses were also made for free phenol groups by the pH 8.0 method.

Diacetyl Tyrosine—To 0.4 gm of crystalline diacetyl tyrosine was added 35 ml

of water containing 0.5 ml. $M/1$ pH 5.0 citrate buffer. To this solution was added with stirring 34 ml. $M/10$ pH 10.5 borate buffer and 1 ml. of 1 N sodium hydroxide the resulting mixture being pH 9.0 by the hydrogen electrode. Aliquots were analyzed from time to time for free acetic acid and free phenol groups by the above mentioned procedures. The reaction mixture was also kept at 35°C.

Rate of Acetylation of Tyrosine and Tryptophane

It seems highly probable from the foregoing experiments that the groups in the protein pepsin which, when acetylated, are responsible for the changes in activity and pH 8.0 chromogenic value, are tyrosine phenol groups. There exists, however, the possibility that the color producing group or structure of tryptophane in the protein pepsin may be acetylated and, when acetylated, causes the change in specific enzymatic activity. It was thought that a comparison of the rate of acetylation of tyrosine and of tryptophane would furnish evidence in this connection. It was decided to attempt acetylation under the conditions used for the acetylation of pepsin, *i.e.*, in strong acetate buffer (pH 5.0–6.0). Since tyrosine is only slightly soluble under these conditions the glycyl derivatives of the two amino acids were used instead. Under the conditions of acetylation (in strong acetate buffer) acetyl figures would have been very difficult. Following the change in chromogenic value of the solution by both the pH 11.0 and pH 8.0 methods serves much the same purpose. By comparing the color values obtained by these two methods one may determine quantitatively the alkali reversible change in color properties of the molecule due to acetylation.

Experimental Procedure

0.5–1.0 gm. of Hoffman-La Roche preparations were dissolved or suspended in approximately 40 ml. of 3 M acetate buffer at the pH indicated. Ketene from the generator previously described (9) and which was used in the preparation of the acetyl pepsin derivatives was passed in slowly with stirring. The materials, if only slightly soluble in the initial stage, were quickly converted (probably by acetylation of the amino groups) into a soluble form. Aliquots were removed at varying intervals of time, diluted, and chromogenic values determined by the pH 8.0 and pH 11.0 methods.

From Table II it is readily seen that there is a gradual decrease in pH 8.0 color value of glycyl tyrosine solution during acetylation, whereas the pH 11.0 color value remains constant. This is inter

puted as acetylation of the phenol group of glycyl tyrosine. The acetyl group is hydrolyzed yielding the full value in the pH 11.0 method, whereas the pH 8.0 method measures only the free or un-acetylated phenol groups. In the case of tryptophane and glycyl tryptophane there is a decrease in color values as measured by both

TABLE II

Acetylation of Glycyl Tyrosine, Glycyl Tryptophane, and Tryptophane by Kclene

Material	pH	Time	Calculated from color value by the		Ratio pH 8.0 value to pH 11.0 value	Acetylated (calculated)
			pH 11.0 method	pH 8.0 method		
		hrs	mg/ml	mg/ml		per cent
Glycyl tyrosine	5-6	0	1.6	1.5	0.94	0
		0.5	1.6	1.2	0.75	20
		1.0	1.6	0.92	0.57	39
		1.5	1.5	0.57	0.38	60
		2.0	1.5	0.47	0.31	67
		3.0	1.4	0.28	0.20	79
Glycyl tryptophane	5-6	0	2.0	1.2	0.60	0
		1.0	1.2	0.76	0.63	0
		2.0	1.1	0.75	0.68	0
		5.0	1.2	0.90	0.73	0
Tryptophane	5-6	0	3.7	3.9	1.1	0
		2.0	2.3	2.8	1.2	0
		4.0	2.1	2.0	1.0	0
Glycyl tyrosine	4.0	0	1.7	1.6	0.94	0
		0.5	1.6	1.4	0.87	7
		1.0	1.7	1.3	0.77	18
		1.5	1.7	1.2	0.71	24
		2.0	1.6	1.1	0.69	26
		3.0	1.6	1.0	0.63	33

methods. The ratio of pH 8.0 color to pH 11.0 color is practically constant throughout although there is a considerable loss in total color value. The tryptophane solution after 4 hours of acetylation was heated with alkali and yet the change in color value occurring during acetylation did not revert to its original value. The change in color of tryptophane and glycyl tryptophane, whether caused by

acetylation or some other factor, is therefore entirely different from the change which takes place on acetylation of glycyl tyrosine or of pepsin

In the previous paper (1) it was pointed out that the specific activity of pepsin drops much more slowly when acetylation is carried out at pH 4.0-4.5 than when carried out at pH 5.0-6.0. If, then, the change in specific activity during acetylation of pepsin is due to acetylation of the tyrosine phenol group, it might be expected that the phenol group of pure tyrosine or glycyl tyrosine would acetylate more slowly at pH 4.0-4.5 than at pH 5.0-6.0. This was found to be the case as may be seen in Table II. The rate at pH 4.3 is less than one half that at pH 5.6. The results of these two experiments tend to eliminate the possibility of tryptophane and point definitely to tyrosine as being the component of pepsin which, when acetylated, results in a marked decrease in specific activity.

Action of Hydrolytic Enzymes on Acetyl Derivatives of Pepsin

All of the foregoing proofs for the existence of acetylated phenol groups in acetylated derivatives of pepsin have been indirect or by analogy. In hope for a more direct proof an attempt was made to isolate the acetylated tyrosine from the acetyl pepsin derivatives. Since acid and alkali will hydrolyze acetylated phenols it was decided to use enzyme solutions as the hydrolytic agents. Solutions of 10 per cent active, 60 per cent active acetyl pepsins, and pepsin (control) as substrates were hydrolyzed at pH 7.0-7.5 with Fairchild's crude trypsin, Wilson's commercial steapsin, crystalline trypsin and chymotrypsin, with the same enzyme solutions at pH 6.0 after heat denaturation of the pepsins, and with Parke Davis 1:10,000 pepsin and crystalline pepsin at pH 2.0 after denaturation, and with the preceding enzymes at pH 6.0 in the native state. During the experiments the chromogenic values of the solutions were followed by the pH 11.0 and pH 8.0 methods and in the last instance ("native" pepsin and acetyl derivatives at pH 6.0) the specific enzymatic activity was followed. Hydrolysis by Fairchild's trypsin of the three different enzyme preparations was attempted at 5°C, 20-25°C (room temperature), and 35°C.

The results were completely negative with regard to isolating any

only 59 per cent of the total number of these groups in protein react at pH 8.0 but with several proteins containing different amounts of tyrosine and tryptophane this interpretation seems unlikely. As yet the writer has no decisive experimental evidence on the question. The interpretation, however, affects only the exact value and does not change the order of magnitude of the final figure.

Amino acid analyses of crystalline pepsin show about 10.3 per cent tyrosine and 2.2 per cent tryptophane.⁶ In order to have a common basis for color giving groups the tryptophane value may be expressed in terms of tyrosine. Thus, 2.2 per cent tryptophane is equivalent to about 2.0 per cent tyrosine. The tyrosine-tryptophane content of pepsin, expressed in terms of tyrosine is, therefore, 12.3

TABLE III

Tyrosine-Tryptophane Content of Several Proteins as Determined by the pH 8.0 Colorimetric Method and the Usual Method after Acid Hydrolysis

Protein	pH 8.0 method	Analysis after acid hydrolysis			Ratio Tyrosine + tryptophane content by pH 8.0 method Tyrosine + tryptophane content after hydrolysis
		Tyrosine	Tryptophane	Tyrosine + tryptophane expressed as tyrosine	
	per cent	per cent	per cent	per cent	
Dialyzed 5 x cryst. P D pepsin	7.9	10.3*	2.2*	12.3	0.64
Dialyzed 3 x cryst. egg albumin	2.9	4.0†	1.2†	5.1	0.57
Kahlbaum-Hammarsten casein	4.1	6.4†	1.4†	7.6	0.54
Horse serum albumin	3.0	4.7†	0.5†	5.1	0.59
					Average 0.59 ± 0.03

* Personal communication of Dr. H. O. Calvery

† Analyses by Folin and associates

per cent or 25 tyrosine groups per mole of pepsin. The tyrosine-tryptophane content of pepsin, as determined by the pH 8.0 method is about 7.7 per cent. Assuming that all the groups react under these conditions, one mol of tyrosine or tryptophane in pepsin gives the color equivalent of $\frac{7.7}{12.3} = 0.64$ mols of tyrosine. In determining the tyrosine and tryptophane content of pepsin at pH 8.0, therefore, the total tyrosine-tryptophane is calculated as $\frac{1.0}{0.64} = 1.6$ times the quantity

⁶ A personal communication from Dr. H. O. Calvery of the Department of Physiological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan.

of tyrosine which develops the same color as the pepsin. The results are shown in Table I.

It may be seen in Table I that the 10 per cent active and 60 per cent active acetyl pepsins have tyrosine-tryptophane values by the pH 8.0 method less than that of pepsin. If these materials are all titrated to pH 11.0-12.0, left for a moment, and then acidified to pH 8.0 followed by an estimation of the color by the pH 8.0 method, they will all show the same value as pepsin. This scheme has been used in this work and has been designated as the pH 11.0 method. The alkali

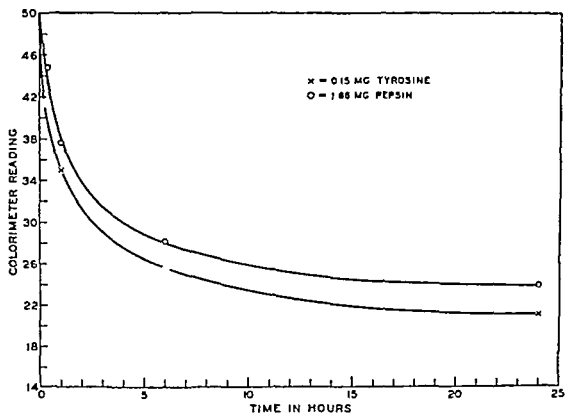


FIG. 3 Rate of color development of pepsin and pure tyrosine by pH 8.0 method

at pH 11.0-12.0 quickly hydrolyzes the acetylated tyrosine phenol groups thus returning the number of pH 8.0 color giving groups to that of pepsin.

In both the pH 8.0 and pH 11.0 methods the phenol reagent is added to the protein solution before the alkaline buffer is added to prevent the solution ever getting above pH 8.0. The color develops slowly at pH 8.0 and reaches a maximum in 10-24 hours at room temperature. As may be seen from Fig. 3 the rate of color development is the same in the protein pepsin as in pure tyrosine. One is justified, therefore, in comparing the colors produced by the protein and by tyrosine at any arbitrary time interval as long as it is the same for both materials. A time interval of 15 minutes has been used during which time the flasks containing the colored solutions were kept at 35.5°C.

Experimental Procedure

The procedure used in the experiment, shown graphically in Fig 3 was as follows 1.86 mg of purified pepsin and 0.15 mg of tyrosine respectively were added to two 50 ml Erlenmeyer flasks and diluted with water to a volume of 17 ml, followed by 3 ml of 1/3 diluted Folin's phenol reagent and 5 ml of the alkaline phosphate solution (60 ml 0.5 M K_2HPO_4 + 34 ml N/1 NaOH + 6 ml H_2O) The alkaline phosphate was added to the flask with stirring by whirling the flask. The solutions were allowed to remain at room temperature and from time to time were read in the colorimeter against a standard blue glass

pH 8.0 Method—An amount of material yielding a colorimeter reading approximately equal to that produced by 0.30 mg of tyrosine under similar conditions is diluted to 11 ml with water. To this solution is added 6 ml of N/10 sodium chloride solution and 3 ml of a 1/3 dilution of Folin's phenol reagent followed by 5 ml of an alkali phosphate. (The alkali phosphate solution is made up of 60 ml of 0.5 M K_2HPO_4 + 34 ml N/1 NaOH + 6 ml of H_2O) This solution is placed at 35.5°C for 15 minutes and compared to a solution of 0.30 mg of tyrosine under similar conditions. If the amount of salts, buffers, and non-protein nitrogen content of the original material to be tested is very small the pH of the final colored solution will be $pH\ 7.8 \pm 0.2$. If any buffer or alkali neutralizing material be present to any appreciable extent (which should be determined before the estimation by simply titrating an aliquot) a determined amount of alkali should be added to the 5 ml of alkali phosphate to bring the solution to the same pH in all measurements. Approximately 3.0 mg of pepsin protein is used in the pH 8.0 method of estimating tyrosine-tryptophane values. Aliquots of a standard solution of tyrosine were run parallel to the protein solutions using the same technique and reagents.

pH 11.0 Method—An amount of material yielding a colorimeter reading approximately equal to that produced by 0.30 mg of tyrosine under similar conditions is diluted to 11 ml with water. To this solution is added 3 ml of N/10 sodium hydroxide and the solution allowed to stand about 5 minutes and then the alkali is neutralized by 3 ml of N/10 hydrochloric acid. 3 ml of 1/3 dilution of Folin's phenol reagent is added followed by the introduction of 5 ml of the alkali phosphate solution described in the pH 8.0 method. The 3 ml of N/10 sodium hydroxide is sufficiently strong (unless buffers are present) to carry the pH of the solution to or beyond pH 11.0 where the acetyl groups come off of the phenol groups almost instantaneously.⁷

⁷ In some of the experiments reported in this paper the procedure of the pH 11.0 method was not identical with that described above. The color was allowed to develop at pH 11.0–12.0 rather than at pH 8.0. The results obtained in this way, although different from the results by the above described pH 11.0 method, were proportionately different for all the chromogenic materials used and so the end result was not affected. The pH 11.0 method, as above described, was later developed and is to be preferred because of its general convenience.

SUMMARY

Crystalline 60 per cent active acetyl pepsin has 7 acetyl groups per mol of pepsin, 3 of which are readily hydrolyzed in acid at pH 0.0 or in weak alkali at pH 10.0

The tyrosine tryptophane content of this acetylated pepsin, measured colorimetrically, is less than pepsin by three tyrosine equivalents

Hydrolysis at pH 0.0 or pH 10.0 of the 3 acetyl groups results in a concomitant increase in the number of tyrosine equivalents. In the pH 0.0 hydrolysis experiment there is also a simultaneous increase in specific activity

The phenol group of glycyl tyrosine is acetylated by ketene under the conditions used in the acetylation of pepsin and the effect of pH on the rate of acetylation is similar in the two cases

It is concluded that the acetyl groups in the 60 per cent active acetyl pepsin, which are responsible for the decrease in specific enzymatic activity, are 3 in number and are attached to 3 tyrosine phenol groups of the pepsin molecule

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STUDIES ON BIOLUMINESCENCE

II THE PARTIAL PURIFICATION OF CYPRIDINA LUCIFERIN

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Kanda has used several methods for purifying *Cypridina* luciferin (1-3), from the last of which he has reported a crystalline product. His method (2) when repeated with 10 gm lots concentrated the luciferin from 15 to 30 times. His last method (3) was less successful because most of the luciferin was extracted from the final benzene solution or oxidized, by the recommended washing with "large amounts" of water. By making use of this extractability of luciferin from benzene solutions or residues by 0.5 M hydrochloric acid instead of water, particularly in the presence of small amounts of ethyl alcohol, and the 10 or 20 to 1 distribution ratio of luciferin between N-butyl alcohol and the acid, preparations 200 or 300 times as active as the initial dry *Cypridina* were obtained. Results were similar when the luciferin was precipitated from the dilute acid solutions, alcohol free, with an excess of flavianic acid. However, losses from oxidation and incomplete separations were large. Also, although Kanda considered the benzene solubility as good support for his suggestion that luciferin is a phospholipid, it would go into benzene only under limited conditions and much of the behavior indicated that other materials present played an important part both in the transfer into and out of benzene.¹

¹ For instance when a benzene solution was shaken with an equal volume of HCl, no alcohol being present, less than half of the luciferin transferred to the HCl during a few minutes of shaking. However, when once in the HCl no appreciable quantities have ever been found to transfer back from it into pure benzene. On the other hand although luciferin is extracted much more slowly from the original powder by alcohol than by water, when once extracted the greater solubility in any of the lower alcohols compared with benzene ether or aqueous solutions is the most striking solubility characteristic up to any degree of purity so far obtained.

The idea of purifying oxyluciferin (4-6) failed when the several attempts to reduce it with H_2 and platinized asbestos or sodium hydrosulfite ($Na_2S_2O_4$), produced a very small percentage of reduction. In HCl solutions, using $Na_2S_2O_4$, considerable apparent reversal was finally obtained but only within a narrow range of reagent concentrations and when the luciferin was in the oxidizing medium, ceric sulfate, for about a minute or less.

A more successful approach was opened by preparing apparent derivatives of luciferin, more stable in the presence of oxygen than luciferin itself. Under some conditions either acetic anhydride or benzoyl chloride produced such, largely inactive, forms of luciferin. Most of the acetylated luciferin was reactivated by cold 0.5 M HCl. Up to 86 per cent of the benzoylated luciferin was reactivated by hot 0.5 M HCl in the absence of oxygen but in a procedure certainly permitting some losses.² The properties of this latter compound have made it very useful in the purification work. For the rest, conditions and solvents have been chosen that have been found to allow a minimum velocity of oxidation of the free luciferin.³

Procedure

The method of estimating the quantity of luciferin present was that previously described (7) except that runs to be compared were made in the presence of a constant amount of sodium chloride. This was found desirable because of the

² Although it is realized that absolute proof of compound formation has not been obtained, the evidence presented by the reversible inactivation of the light-producing substrate by two compounds under conditions where an active hydrogen would be removed and returned, the striking apparent change in solubility, and the greatly increased resistance to oxidation by atmospheric oxygen, is sufficiently strong to justify thinking of it in those terms until something inconsistent with such a view appears.

³ The method of extraction, the degree of purity, and in some cases the concentration of luciferin were found to influence enormously its rate of oxidation in a given solvent. In addition smaller variations of unknown origin were apt to occur. The rate of oxidation previously given (7) for certain hydrochloric acid solutions is, therefore, not general. Some experiments indicated that at least a part of this complicated behavior was due to other materials present in the various preparations. That the rate of loss of luciferin was actually sensitive to some compounds was shown by the great increase in rate obtained after the addition of small amounts of hydroquinone and especially cuprous chloride to certain solutions.

surprisingly large specific effect of very small concentrations of sodium chloride on the total light emitted, in spite of the 0.067 M phosphate always present in the reaction mixture. The final concentration, including any sodium chloride formed from the neutralization of hydrochloric acid, was usually about 0.011 M. The concentration of luciferase added was 0.8 per cent throughout. The volumes of luciferin used were such as to make the total light emitted by samples from different solutions of the same order of magnitude.

The whole, dry organisms were powdered in a ball mill and extracted in a Soxhlet with ether and then with benzene. The luciferin remained in the solid and was stable in this condition in the absence of water.

Since the quantity of material is limited, originally only 10 gm. lots were used from this point on. Later this was increased to 50 gm. with the same procedure except as indicated.

Two different methods of extraction of luciferin from this preextracted material have been used successfully on the small lots. In the first method the *Cypridina* powder was placed in a Buchner funnel and the luciferin extracted by slowly pouring through it 300–400 ml. of a boiling 0.1 M NaCl solution containing 10 per cent ethyl alcohol, keeping the solid covered with liquid at all times. The filtrate containing the luciferin, filtered into a chilled mixture of 2.5 M HCl and butyl alcohol. This is a modification of Harvey's original method. The ethyl alcohol increased the amount extracted and the rest of the procedure quickly transferred the luciferin from the extremely unstable hot aqueous solution to the much more stable cold acid or butyl alcohol solution. The second method was to follow Kanda and extract with methyl alcohol. This was slower but gave somewhat better yields and was less liable to accidents. It was used for all of the later work.

The extractions were made with from 5 to 10 ml. of methyl alcohol per gram of *Cypridina* powder and continued under hydrogen for about 24 hours. The solution was filtered and the residue washed with methyl alcohol. This step must be rapid as considerable losses, particularly with the 50 gm. lots occurred here. 25 ml. of N butyl alcohol were added to the filtrate and the methyl alcohol removed from the deaerated solution *in vacuo* at room temperature. The residual liquid was decanted from the precipitate which formed and the precipitate washed with several 15 ml. portions of butyl alcohol. The suspension was centrifuged down each time. Since it was difficult to wash this precipitate free of luciferin it was sometimes dissolved in 0.5 M HCl and the luciferin extracted from that solution with butyl alcohol. This was easier and faster but considerably more of a red pigment was carried into the butyl alcohol. The mixed butyl alcohol extractions, totaling 50 or 60 ml. were chilled and then benzoylated with 2 ml. of benzoyl chloride. The relatively unfavorable conditions for benzoylation were probably an advantage from the purification standpoint. After 15 minutes in the ice bath the solution was tested for free luciferin. If it was present to more than 1 or 2 per cent, more benzoyl chloride was added. If not the solution was washed with three successive equal volumes of water during about one half hour to allow time for the excess benzoyl chloride to hydrolyze. The butyl alcohol fraction

was then dissolved in ten volumes of water. A highly colored material, apparently dissolved in the butyl benzoate formed in the above reaction, remained as a separate phase. The inactive luciferin in this suspension was then extracted with 80 ml of ether⁴ followed by three portions of 40 ml each. When a stable emulsion formed, as was frequently true during the first extraction, it was broken by centrifuging. The several ether fractions were mixed and the ether removed *in vacuo* without deaeration. The residual solution of inactive luciferin in butyl alcohol was then mixed with 250 ml of 0.55 M HCl and carefully deaerated. This suspension was heated with a water bath kept between 95–100° for 1 hour and then cooled in an ice bath. The hydrogen was left bubbling through during these operations. When thoroughly cooled, the mixture was washed with ether as above except that centrifuging was not ordinarily necessary. Most of the pigment, which was concentrated in a small film on top of the aqueous solution, went into the ether. Most of the luciferin remained in the aqueous phase although up to 4 per cent appeared in the first ether washing. No significant amounts appeared in the later washings. These washings also removed the butyl benzoate and the benzoic acid, formed from the excess benzoyl chloride, quite completely from the aqueous phase. The luciferin was extracted from the HCl solution by 40 ml of N-butyl alcohol followed by four portions of 20 ml each. The benzoylation and hydrolysis were then repeated in essentially the same way. Sometimes more benzoyl chloride was required the second time because of a greater volume of butyl alcohol and the presence of some HCl.

Where obvious accidents had not occurred about an 80 per cent yield was obtained for each complete cycle of benzoylation, hydrolysis, and return to butyl alcohol. Apart from that mentioned above, no discarded fractions contained more than 1 or 2 per cent of the luciferin present. Much of the loss was probably from oxidation although some may have occurred during the heating with HCl.

The final butyl alcohol solutions were yellow, as were many of the discarded fractions, and had an activity in arbitrary light units⁵ of from 40,000 to 60,000 per gm of dry weight. After one benzoylation cycle the activity was from 13,000 to 30,000 units. The dry *Cypridina* used for these preparations had in the same units an activity from 21 to 33. This was based on the most active initial methyl alcohol extracts obtained from small samples of a given bottle of material.

⁴ Great care must be taken to avoid oxidizing agents in the ether which may easily attain a sufficient concentration to destroy much of the luciferin. Some lots may be kept safely for months after opening, while others apparently must be purified the same day as used.

⁵ This unit was a reading of 1 volt on the potentiometer in the particular experimental arrangement previously described (7).

SUMMARY

Some solubility, oxidation, reduction, and compound forming characteristics of extracts of *Cypridina* luciferin have been presented

A method of purification has been described which increased the amount of luciferin per unit of dry weight, as measured by the total light emitted, to about two thousand times that in the dry starting material. The best yields were from 50 to 65 per cent.

I wish to thank Professor E. Newton Harvey for his support of this work.

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PACEMAKERS IN NITELLA

I TEMPORARY LOCAL DIFFERENCES IN RHYTHM

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(Accepted for publication May 22 1935)

Theoretically a pacemaker can be produced at any spot where the ρD can be sufficiently reduced and a sharp potential gradient can be maintained. This can be done experimentally¹ by means of KCl chloroform, or ethyl alcohol.

Pacemakers occur spontaneously in many cases and may produce a variety of rhythms. In studying these we have observed a phenomenon which recalls a well known feature of auricular flutter in the heart, *i.e.* when the auricle beats 300 times per minute the ventricle may beat at half that rate.² In this case every other negative variation of the auricle is registered in the ventricle.

The situation in *Nitella* is shown in Fig. 1. The cell was arranged as in Fig. 2. A series of negative variations originating spontaneously from a pacemaker near the right end of the cell passed in succession through *E*, *D*, and *C*. The negative variations at these spots are recorded by separate strings and are shown on the record at *C*, *D* and *E*.³

The record begins at the point marked 1 with a monophasic negative variation shown by the upward and downward⁴ movement of the action curve at *E*, followed by similar movements at *D* and *C*.

After a second variation a break occurs at 2. At 3 two more move

¹ Osterhout W J V and Harris E S *J Gen Physiol* 1921-28 11, 673.
Osterhout W J V and Hill S E *J Gen Physiol* 1930-31 14, 611.

Osterhout W J V and Hill S E *J Gen Physiol* 1934-35 18, 499.

² Lewis T. Clinical electrocardiography. London: Shaw and Sons, 1913, 84.

³ Regarding material and technique see Osterhout W J V and Hill S E *J Gen Physiol* 1933-34 17, 87; 1934-35 18, 499.

⁴ See Hill S E and Osterhout W J V *J Gen Physiol* 1934-35 18, 317.

ments occur at *E*, followed by single movements at *D* and *C*. In the movements starting at 4, each movement at *E* is followed by one at *D* and *C*.

Such a state of affairs may evidently depend on a change in the refractory period. It has been suggested⁶ that the refractory period depends largely on the time required to move back into the sap the potassium which has moved out during the action current. This in turn would depend on the permeability of the protoplasm and the forces producing the movement of potassium; these forces are of course derived from metabolism.⁷

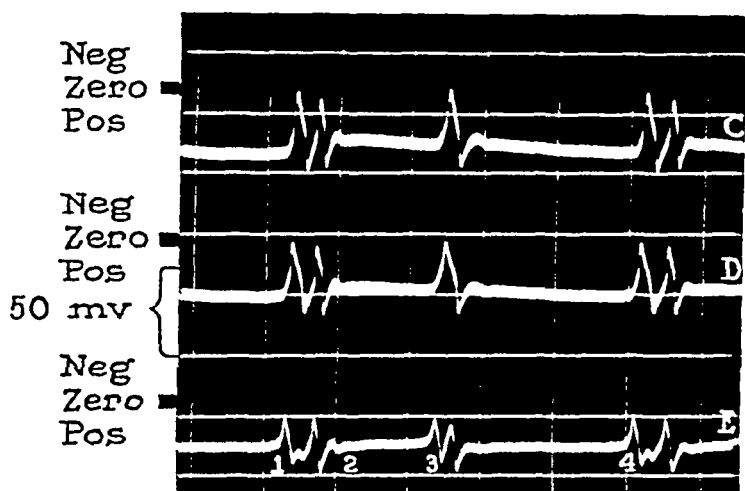


FIG. 1. Photographic record of negative variations; the cell being arranged as in Fig. 2.

A series of negative variations originating near the right end of the cell passed in succession through *E*, *D*, and *C* (Fig. 2). The variations at these spots are recorded by separate strings and are shown on the record at *C*, *D*, and *E*. The record begins at the point marked 1, with a monophasic negative variation shown by the upward and downward movement of the action curve at *E*, followed by similar movements at *D* and *C*. After a second variation a break occurs at 2. At 3 two more movements occur at *E*, followed by single movements at *D* and *C*. In the movements starting at 4, each movement at *E* is followed by one at *D* and at *C*.

Room temperature 22°C. The vertical lines are 5 seconds apart.

⁶ Osterhout, W. J. V. *J. Gen. Physiol.* 1954-55, 18, 215.

⁷ We must consider the possibility of a conflict of pacemakers but there is no evidence of such a conflict in the present case.

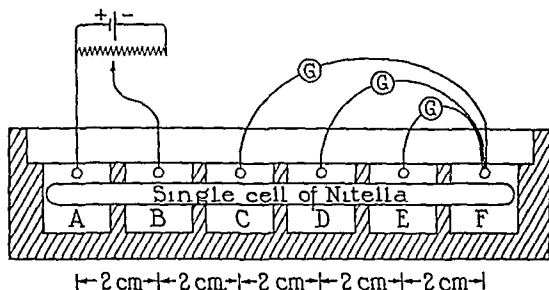


FIG 2 Diagram of a series of paraffin cups *A* to *F*, with a single cell of *Nitella* passing through all of them. *GGG* represent string galvanometers (three strings inserted in the single magnetic field of a Type A Cambridge string galvanometer) with vacuum tube amplifiers, arranged as short period voltmeters. Ag AgCl electrodes dip into the cups. Cf Osterhout, W J V, and Hill, S E, *J Gen Physiol*, 1933-34, 17, 87. *A*, *B*, *C*, *D*, and *E* are in contact with 0.01 M NaCl. *F* is in contact with 0.01 M KCl. The cell had been kept for 2 hours in 0.01 M NaCl before use.

SUMMARY

A series of negative variations passing along the cell may reach a region where only every other variation registers. This condition may be temporary. It would seem to depend on a local change in the refractory period.

THE VISUAL ACUITY OF THE FIDDLER-CRAB, UCA PUGNAX*

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(Accepted for publication, May 1, 1935)

Visual acuity in the human eye varies in a specific way with the illumination. The relation between the visual acuity and the logarithm of the intensity is described by a sigmoid curve (Koenig, 1897, Hecht, 1927-1928). Hecht and Wolf (1929) presented the first quantitative data on the relation between visual acuity and illumination in an animal with compound eyes. They used an experimental procedure involving neither conditioned reflexes nor training. Their data show that despite the morphological differences between the vertebrate and the arthropod eye a similar relation exists between visual acuity and illumination in the honey bee and man. *Drosophila* was found to show a like relation by Hecht and Wald (1934).

The importance of such results indicates the advisability of extending the study to other animals. Hecht and Wolf, starting with the common observation that most animals with eyes respond to a sudden movement in their visual field, developed a method of measuring visual acuity quantitatively.

Observation showed that movement in the visual field above a fiddler-crab elicited a response. Application of the reasoning and methods of Hecht and Wolf indicated a method by which quantitative data might be secured.

This paper reports a study of visual acuity in the fiddler crab. It involves an investigation of monocular and binocular visual acuity, and analysis of the responses to a visual pattern in terms of the structure of the eye, and finally, field observations which attempt to confirm the laboratory findings.

* Investigation pursued during tenure of a National Research Fellowship

Nature of Response

The experimental procedure with bees and *Drosophila* consists in moving a pattern made up of alternate dark and light bars in the visual field of the animal. It was found that the normal fiddler-crab, while giving uniform and consistent responses to a pattern plate made up of one dark bar on a white background, gives inconsistent, indeterminate, or no response to a pattern plate made up of alternate dark and white bars such as was used in the experiments with bees and *Drosophila*.

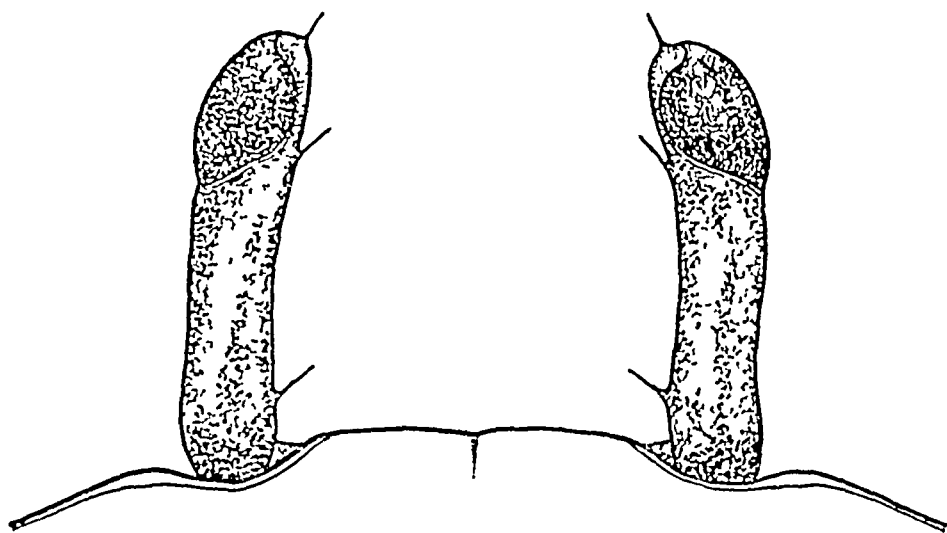


FIG 1 View of the eye stalks and ommatidia in the fiddler-crab. Note that the ommatidia are found only on the distal outer side of each stalk.

An examination of the ommatidia on the eye stalks of the crab and an analysis of the response provides the explanation. The ommatidia are arranged only on the distal outer side of each eye stalk as shown in Fig 1. Thus there is little or no overlapping of the visual fields of the two eyes. When a pattern plate composed of a single stripe is passed over a fiddler-crab the following reactions may occur. (A) If the stripe is moved slowly, the crab will move away, keeping in front of the stripe continuously. (B) If the stripe is moved so as to overtake and pass the crab, the crab will move ahead of the stripe until the stripe passes over it, at which time the crab will stop and

move in the opposite direction (C) If the stripe is moved rapidly the two movements of the crab as noted above in (B) occur so rapidly that the crab appears to "jump"

The three responses are not separate types, but are variations due to the speed at which the stripe is moved. If the stripe is moved from the animal's right to its left to give type (B) response, the crab will move first to the left then to the right. If the stripe is moved from the animal's left to its right, the direction is reversed. It seems that a moving object stimulating the right eye (irrespective of direction of movement) will cause the crab to move to the left, while stimulation of the left eye will cause the crab to move to the right. If this is true then a pattern plate made up of stripes in passing over a fiddler crab would of necessity stimulate both eyes, and tend to cause movements in opposite directions. It may well be that under such conditions the stimuli inhibit each other with the result that little or no response appears.

Corroboration is given to the above explanation by the behavior of fiddler crabs with one eye extirpated or removed. If the right eye is blinded and the pattern plate is moved from the left to the right, the animal will move to the right until the stripe passes over it, whereupon it will stop. If the pattern plate is moved from the right, no response is elicited until the stripe passes over the animal, which then moves to the right. If the left eye is blinded the response is similar but opposite in sign. In other words if one eye is blinded part of the normal response to the pattern plate disappears as would be expected.

Apparatus and Procedure

A thousand watt lamp (Fig. 2) enclosed in a double lamp-box was used to radiate light through a water cell and a neutral tint wedge on to a mirror placed at an angle of 45° to the beam of light. The light was reflected downward by the mirror on to an opal glass plate. Immediately beneath was the pattern plate made by inserting a strip of black paper between two plates of glass, thus giving a pattern of a single black bar on a transparent field. Fifteen pattern plates with stripes of different size were made. A frame which would move freely in a pair of grooves held the pattern plate. The movement of the frame with the plate constituted a moving visual pattern.

The fiddler-crab was confined in a glass bottomed compartment, 10×10 cm in area directly below the pattern plate. A mirror placed below the animal compartment gave a view of the crab and the visual field.

The intensity of the illumination of the visual field was varied by movement of a calibrated neutral tint wedge. By addition of neutral filters the intensity could be varied over a range of 1:100,000.

The procedure for making a measurement was as follows. A pattern plate was inserted in the movable frame. A fiddler-crab which had been adapted overnight was placed in the animal compartment under a low illumination and left until it became quiet. Then the pattern plate was moved with a sharp but not rapid movement. If no response was elicited it was assumed that the animal could not distinguish the components of the visual pattern and the illumination was increased. If the displacement of the pattern did cause a response the intensity was lowered.

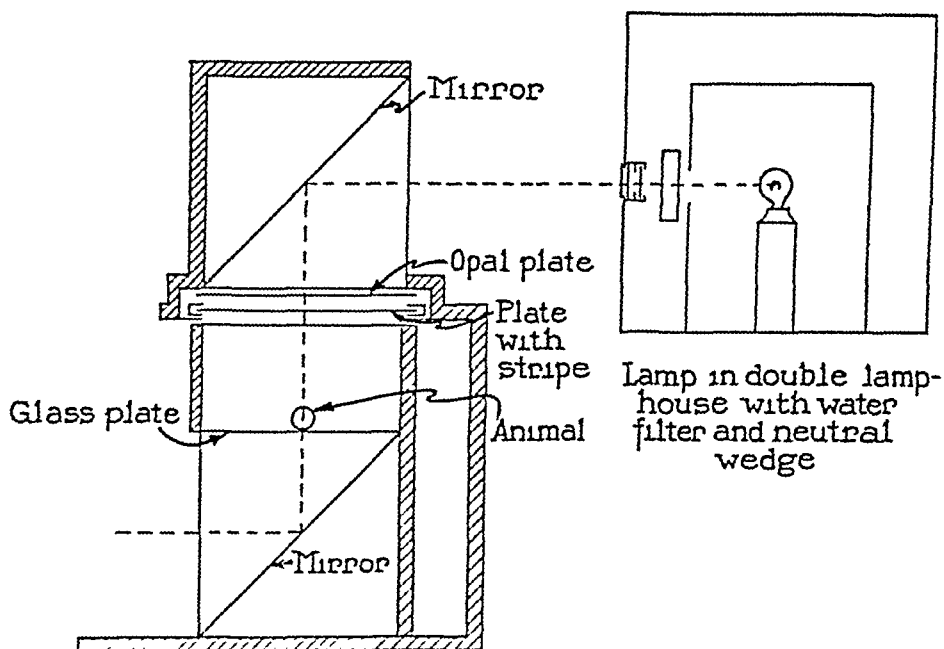


FIG. 2. Apparatus for measuring the visual acuity of the fiddler-crab.

This procedure was repeated until the minimum illumination was found at which the crab would react to a displacement of the field. The visual acuities secured by the use of the plates depend on the width of the stripes and their distance from the eye of the crab. The distance was measured for each crab, because of the difference in size of the crabs, the size of the bar being known, the resulting angle in minutes and its reciprocal—the visual acuity—could then be computed.

RESULTS

Measurements for each of eighteen normal crabs were made with most of the fifteen pattern plates. It is a simple matter to cover or

extirpate one eye of the fiddler crab. Measurements of monocular visual acuity were made in the same apparatus with seven crabs using six pattern plates.

The averaged data are given in Table I and are represented graphically in Fig. 3, where each point is an individual reading showing the actual visual acuity and minimum intensity to secure a reaction. It is immediately evident that the results are similar for both monocular and binocular vision. The data present a relation between visual acuity and intensity which is very similar to the sigmoid curve for

TABLE I

Relation between Visual Acuity and Intensity of Illumination for Fiddler Crabs

With both eyes		With one eye	
Intensity	Visual acuity $\times 10^4$	Intensity	Visual acuity $\times 10^4$
<i>foot candles</i>		<i>foot candles</i>	
0.124	3.91		
0.283	5.07	0.269	4.90
0.676	6.76		
0.723	9.87	1.32	9.73
1.70	12.62		
2.80	15.42		
3.41	19.59	4.30	19.60
4.49	23.32		
6.93	26.20		
13.8	29.25		
27.5	34.05	19.5	33.69
138.0	38.90	51.3	38.51
263.0	42.03	208.9	42.31

the human eye and the honey bee. The curve drawn through the data is the stationary state equation, $0.16 I = (x-3)/(42-x)$, where x is the visual acuity multiplied by 10,000. This gives a maximum visual acuity of 42×10^{-4} for the fiddler crab.

Field Observations

An attempt was made to verify in the field the laboratory results on maximum visual acuity. It is commonly observed that when a person passes over a flat terrain on which there are numerous fiddler crabs, they will quickly vacate a zone around the intruder. This zone

remains fairly constant in radius and moves as the intruder moves, the fiddler-crabs either seek holes or move away. This phenomenon has been variously explained as the result of vibrations set up in the ground, as a visual response, or even as being chemical in nature. It seems most probable that the response is visual, and if so, it should be possible to test the maximum visual acuity in terms of the following considerations

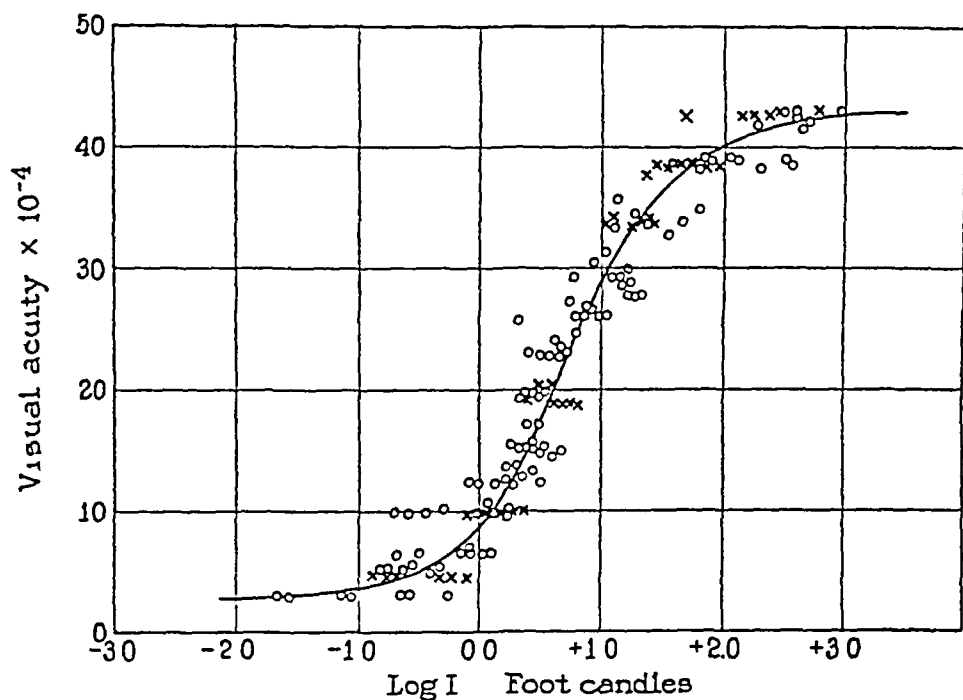


FIG 3 Relation between visual acuity and illumination in fiddler-crabs. Circles are readings secured from normal crabs, and crosses are readings from crabs with one eye covered or extirpated. The points are individual readings.

Fiddler-crabs will retire from a moving object until they can no longer see it, i.e. until such time as the angle subtended by the object is smaller than the smallest effective visual angle, then the crabs will stop or move at random. Therefore the radius of the observed clear zone is a measure of the maximum distance at which the crabs can see a particular moving object. If the size of the object and the distance of the nearest crabs are known the minimum visual angle and thus the maximum visual acuity can be determined.

One of the mud flats near Hillview, Staten Island, proved satisfactory for the experiment. In the early spring the vegetation had been burned off, fiddler crabs were numerous and well distributed, and the terrain was flat with no large obstructions.

On a day on which the illumination was at least 4500 ft candles and consequently not the limiting factor, tests were made by carrying black screens 20, 40, and 60 inches in width into the field. Five observations were made of the width of the cleared zone with each screen. The results in each series were averaged and the minimum visual angle and maximum visual acuity determined. These gave visual acuities of 0.0041 for the 20 inch screen, 0.0038 for the 40 inch screen, and

TABLE II

Average Angular Distance Apart of Ommatidia in Longitudinal Section of Eye of Fiddler Crab (Ommatidia Numbered from Distal to Proximal End of Eye)

Ommatidial No	Average angular distance	Ommatidial No	Average angular distance	Ommatidial No	Average angular distance
	degrees		degrees		degrees
1-4	18.33	25-28	3.0	49-52	4.47 (?)
5-8	11.78	29-32	2.88	53-56	4.43 (?)
9-12	7.37	33-36	2.05	57-60	4.75
13-16	6.27	37-40	2.82	61-64	5.43
17-20	5.88	41-44	2.90	65-68	5.97
21-24	4.05	45-48	2.89	69-72	6.27

0.0032 for the 60 inch screen. Although they do not compare exactly with the laboratory maximum visual acuity of 0.0043, they are of the same order of magnitude. The low visual acuity from all screens and particularly of the large one may well be due to small obstructions preventing a clear visual field to the crabs.

Relation between Visual Acuity and the Internal Structure of the Eye

The analysis of the variation in visual acuity with illumination for man by Hecht (1927-28), for the bee (Hecht and Wolf, 1929), and *Drosophila* (Hecht and Wald, 1934) in terms of the distribution of the functional receptors is supported by the work on the fiddler-crab. In man and the bee the minimum visual angle (which determines the

maximum visual acuity) is equal to the minimum angular distance between two adjacent receptors. In *Drosophila* the minimum visual angle (9.28°) is twice that of the minimum angle (4.2°) between adjacent ommatidia.

To determine the situation in the fiddler-crab longitudinal sections of the eye were made and the angular distances between the ommatidia measured. There are approximately 72 ommatidia in longitudinal section and their average angular distance apart from the distal to the proximal part of the eye is given in Table II.

Accordingly, it will be noted there is an area near the center of the eye in which the angular distance between two adjacent ommatidia is considerably smaller than over the rest of the eye. In this area the minimum angular distance between two adjacent ommatidia is 2.05° . It would be expected from Hecht's and Hecht and Wolf's analyses of acuity in man and the bee that the minimum visual angle would be the same, namely 2.05° . Actually the minimum visual angle is 3.87° , approximately twice the minimum angular distance between two adjacent ommatidia. Although experimental error might account for this discrepancy, the similar relation between minimum ommatidial angle and visual acuity found by Hecht and Wald in *Drosophila* (4.2° to 9.28°) suggests that the difference is more fundamental.

SUMMARY

The visual acuity of the fiddler-crab can be measured at various illuminations by means of its response to a moving visual pattern. The method, although similar to that used by Hecht and Wolf for the bee and Hecht and Wald for *Drosophila*, must be modified to give consistent results. An explanation of the response to a visual pattern is given in terms of the structure of the eye.

Visual acuity of the crab varies with $\log I$ as in man, the bee, and *Drosophila*. Hecht and Wolf's explanation of the varying visual acuity with illumination in terms of the distribution of functional ommatidia in the eye is supported to that extent.

In the fiddler-crab as in man, monocular and binocular visual acuity is similar with a maximum of 0.0042 for the fiddler-crab. This agrees fairly well with visual acuities of 0.0041, 0.0038, and 0.0032 as found in the field.

In man and the bee, the minimum visual angle corresponds to the minimum angle of two adjacent receptors, in *Drosophila* and the fiddler-crab the minimum visual angle corresponds to approximately twice the minimum angle between two adjacent receptors

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THE DARK ADAPTATION OF RETINAL FIELDS OF DIFFERENT SIZE AND LOCATION*

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(Accepted for publication May 9, 1935)

I

Purpose of Work

The histological structure of the human retina (Rochon Duvidneaud, 1907, Wolfrum, quoted by Dieter, 1924) is such that a central area whose diameter subtends a visual angle of a little less than 2° is practically free of rods and made up almost entirely of cones, whereas retinal areas outside of this restricted region contain rods in addition to the cones. From the center of the retina to its periphery the number of rods increases while the number of cones decreases, so that the ratio of rods to cones in a retinal area of given size increases as the area is moved from the center to the periphery.

It follows from these facts and from von Kries' theory of the separate functions of rods and cones (von Kries, 1929) that measurements of visual capacities should show an almost pure cone behavior when restricted to the rod free 2° central area, and a combination of both rod and cone behavior for larger central areas, or for any areas situated outside the fovea. Numerous researches have shown that this is true for visual acuity (Koenig, 1897), for dark adaptation (Hecht, 1921, Kohlrausch, 1922, Takagi and Kawakami, 1924, Dieter, 1929), for color vision (Koenig, 1903, Dieter, 1924), for flicker (Hecht and Verrijp, 1933), and for intensity discrimination (Hecht, 1934).

The existing measurements of dark adaptation have made it clear that cone and rod adaptation possess distinctly different characters.

* The data and conclusions contained in this paper were presented in preliminary communications to the American Physiological Society in the spring of 1932 (*Am J Physiol* 1932, 101, 52) and to the XIV International Physiological Congress in Rome in the summer of 1932 (*Arch sc biol*, Italy 1933 18, 170).

tics Cone dark adaptation as ordinarily measured (Hecht, 1921, Kohlrausch, 1922, Dieter, 1929) is (a) limited in extent, covering at most an intensity range of 100 to 1, and (b) rapid in speed, being practically complete in the fovea in 3 minutes. On the other hand, rod dark adaptation (Piper, 1903, Kohlrausch, 1922) is (a) extensive, covering an intensity range of 10,000 to 1, and (b) slow, being practically complete only in about 30 minutes.

Working with a very small area situated 5° above the fovea, Kohlrausch (1922, 1931) has measured its dark adaptation with differently colored lights and has found that in such a retinal region containing rods and cones, the course of adaptation shows the presence of both functions, and that the relative extent of the two is determined by the color of the light, according to the relative spectral sensibilities of the two types of element.

Because the retina varies so strikingly in rod-cone population at different points, it seemed to us that even with white light alone, we could vary the relative contribution of rods and cones to the course of dark adaptation by properly choosing the size and location of the retinal area used for measurement. We present here the results of our measurements of dark adaptation, first with centrally located areas of increasing size, and second with peripherally situated areas of different size and location so chosen as to elucidate the behavior of the retina as a whole.

II

Apparatus and Procedure

The arrangements for making the measurements are shown diagrammatically in top view in Fig. 1. The source of light is a 3.8 volt flash light lamp kept at 0.28 amperes. It illuminates an opal glass plate which serves as the test-field whose brightness is controlled by neutral decimal filters and by a neutral wedge with a transmission range of 1/1000. The filters and wedge were calibrated with a Macbeth illuminometer. The size and position of the test-field are fixed by metal diaphragms next to the opal glass. A fixation point, whose position and brightness can be controlled independently of the test-field, is reflected into the observer's eye by a very thin glass cover-slip. The field is viewed through the exit pupil 2.85 mm. in diameter, and through the lens L_2 , which assists the accommodation of the observer's eye, since the distance from exit pupil to the test-field is only 10 cm. Lens L_1 serves practically no function when the opal plate is in place. When high intensities are necessary for use with monochromatic filters, the opal

plate is removed and another lens is placed between L_1 and the source. This gives an image of the source at the exit pupil and furnishes about 1000 times as much light as with the arrangement shown in the diagram. The illumination of the field is cut off by a shutter which can be raised and lowered by a knob in the hand of the observer.

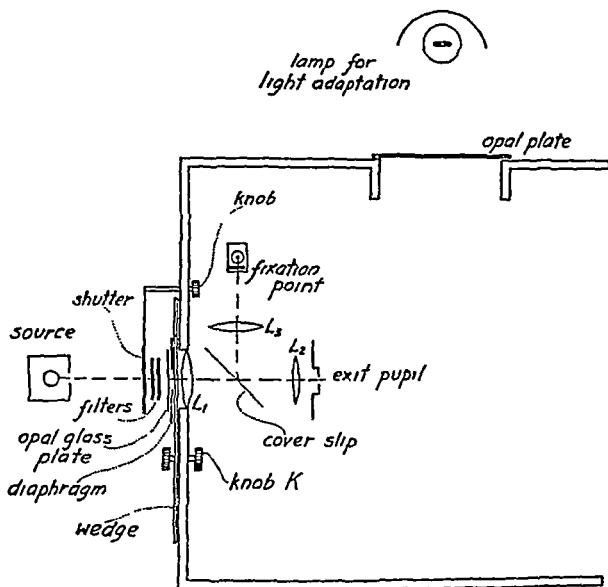


FIG 1 Diagrammatic top-view of apparatus for measuring dark adaptation. For convenience in drawing the fixation point has been placed to the right of the observer; in the set up as used, it is actually above the observer.

A measurement at any moment of dark adaptation consists in the observer's moving the wedge into such a position that the test field just becomes visible. Usually this involves two or three exposures of the field by means of the shutter. More exposures are to be avoided because they raise the threshold perceptibly. The wedge is moved by a rack and pinion controlled by the knob K.

Each run is preceded by 2 minutes light adaptation to a brightness of 300 millilamberts, obtained by placing the eyes 4 cm from an externally illuminated opal glass in the wall of the cubicle. Observations can begin almost immediately after light adaptation, because the moment the adapting light is turned off the observer can turn his head and place his eye at the exit pupil.

In the first few minutes of dark adaptation the threshold drops so precipitously that measurements are made as rapidly as possible. After the first three or four points have been determined, measurements are made more leisurely at intervals of 2 to 5 minutes. The position of the wedge is noted by the recorder in the dark room outside the cubicle. Time is measured with a stop-watch.

III

Centrally Fixated Fields

The measurements were made on the right eye of each of us, we in turn acted as recorders and observers. We have also made occasional runs with various people in the laboratory and with visitors, and are satisfied that our results cover the normal range of variation.

Our first measurements were with centrally located retinal areas 2° , 3° , 5° , 10° , and 20° in diameter. For each area we made at least three runs, frequently five, and occasionally six or seven. Though each individual run is continuous and smooth, its intensity level varies from day to day. Therefore several runs have to be averaged in order that comparisons between the different areas may be made with confidence. We have made some measurements with a 1° centrally fixated field, the results are not much different from a 2° field, but are more irregular, very likely due to the interference of the fixation point (which of course is far from being a point) with the sensitivity and adaptation of so small an area.

The averaged measurements are given in Table I, which is so arranged that comparable times for all areas are on the same horizontal line for each observer. The time in the dark is given in minutes, and the threshold is given as the logarithm of the brightness in micromillilamberts. This unit is one millionth of a millilambert, and was adopted to avoid the fractions and negative logarithms which appear under these conditions with ordinary units like the millilambert. The final threshold of the dark adapted eye for large fields lies between 1 and 10 micromillilamberts.

Inspection of Table I shows that with increasing area the threshold at any moment drops steadily to lower and lower intensity values. However, to gain an adequate idea of the nature and the course of

TABLE I

Centrally Fixated Fields of Different Size Time in Dark in Minutes Intensity in Micromillilamberts

Diameter	2		3		5		10		20	
Observer	Time in dark	Log intensity	Time in dark	Log intensity	Time in dark	Log intensity	Time in dark	Log intensity	Time in dark	Log intensity
S H							0 10	5 11		
	0 20	4 37	0 22	4 25	0 22	4 36	0 20	4 52	0 15	4 34
	0 55	3 98	0 79	3 70	0 81	3 78	0 59	3 88	0 65	3 75
							1 30	3 57		
	2 00	3 82	2 40	3 65	2 20	3 55	2 50	3 49	1 80	3 59
									3 60	3 46
	4 40	3 87	4 70	3 57	4 50	3 36	4 20	3 35	4 80	3 26
									6 70	3 00
	8 30	3 88	8 10	3 54	7 60	3 16	7 20	3 00	7 90	2 57
					10 0	2 97	9 30	2 61	9 40	2 23
	11 8	3 85	12 0	3 51	12 6	2 72	12 3	2 34	11 5	1 97
	15 5	3 76	14 1	3 41	15 1	2 55	15 5	2 12	15 5	1 68
G W			16 3	3 32	18 2	2 45	18 2	1 91		
	20 9	3 71	20 3	3 22	21 9	2 24	21 4	1 82	20 9	1 42
			24 9	3 13			25 1	1 71		
	27 5	3 73	30 2	3 01	28 2	2 13	29 5	1 66	28 2	1 22
	0 20	4 38	0 30	4 33	0 14	4 34	0 23	4 27	0 19	4 34
	0 68	3 96	0 71	3 97	0 54	3 82	0 60	3 76	0 60	3 79
							1 50	3 49	1 40	3 54
	2 10	3 85	2 50	3 64	2 00	3 58	3 00	3 24	2 50	3 23
	4 20	3 95	5 20	3 64	4 30	3 47	4 60	3 11	4 70	3 01
					6 90	3 35	6 50	2 84	6 60	2 64
	7 40	3 94	8 30	3 55			7 90	2 62	8 40	2 21
C. H					9 30	3 14	9 30	2 40		
	11 8	3 83	10 7	3 45	11 5	3 00	10 7	2 28	11 0	1 88
			13 5	3 35	13 8	2 86	12 9	2 10	13 5	1 68
	17 0	3 81	16 6	3 23	17 4	2 63	16 6	1 90	16 6	1 57
	21 9	3 74	20 4	3 14	21 7	2 47	20 0	1 82	19 8	1 39
	27 5	3 67	26 3	3 06	25 1	2 39	24 0	1 78	26 2	1 36
	35 5	3 66	29 5	3 06	31 0	2 34	30 2	1 73		
	0 32	4 37	0 35	4 34	0 22	4 34	0 38	4 27	0 30	4 37
	0 79	3 81	0 74	3 75	0 68	3 84				
					1 10	3 57	0 93	3 73	1 10	3 73
	1 50	3 53	2 00	3 51	1 90	3 39	2 00	3 46	2 20	3 48
	3 20	3 43	3 20	3 36			3 20	3 21		
					4 50	3 16	4 60	2 92	4 30	3 01

TABLE I—*Concluded*

Diameter	2°		3°		5°		10°		20°	
Observer	Time in dark	Log intens sity	Time in dark	Log intens sity	Time in dark	Log intens sity	Time in dark	Log intens sity	Time in dark	Log intens sity
C H—	5 60	3 31	5 40	3 35	6 80	2 73	6 20	2 76		
Concl'd	8 30	3 27	7 90	3 22			7 90	2 45	7 80	2 37
					9 60	2 56	10 2	2 28	9 60	2 00
	12 9	3 22	12 3	3 06	12 6	2 31	12 9	2 06	13 8	1 65
	17 0	3 00	17 8	2 82	15 5	2 15	16 2	1 86	18 6	1 36
	22 4	2 89			19 5	2 05	21 9	1 69	22 9	1 26
			25 7	2 58	24 6	1 94	25 1	1 60		
	28 8	2 91			29 5	1 86	29 5	1 56	29 5	1 19

dark adaptation under these conditions, it is necessary to examine the data graphically. As an example, the measurements of S H are reproduced in Fig 2. This shows that for the 2° field, dark adaptation is rapid and shallow. After 2 minutes, the threshold remains practically constant for about 15 minutes, when there appears a slight additional drop. This second drop is variable in the data of S H, showing up on some days and not on others, it is more regularly present with G W, and always shows up in the data of C H. This secondary drop is always present for all observers with the 3° field, and occurs earlier at about 11 minutes. The significance of the secondary drop becomes clear in the larger fields where, as Fig 2 shows, it appears sooner and goes lower as the field increases in size. For the 5° field the secondary burst of dark adaptation contributes as much to the total range of adaptation as the initially rapid drop in threshold, whereas for the 20° field it contributes about 2½ times as much.

The transition between the primary, comparatively rapid adaptation, and the secondary, more leisurely adaptation is fairly abrupt except for the 20° field where it is more gradual. This is true for S H, for G W, and for all other people whose adaptation we have measured. For C H the transition is gradual even for the 10° field, this being due to the fact that the primary decrease in threshold for C H is slower than for the rest of us. The abrupt transition appears in some of the older measurements of Piper (1903), but the

recognition of its presence and its significance is due to Kohlrausch (1922), who showed definitely that the rapid primary adaptation is due to the cones, and the slower secondary adaptation to rods

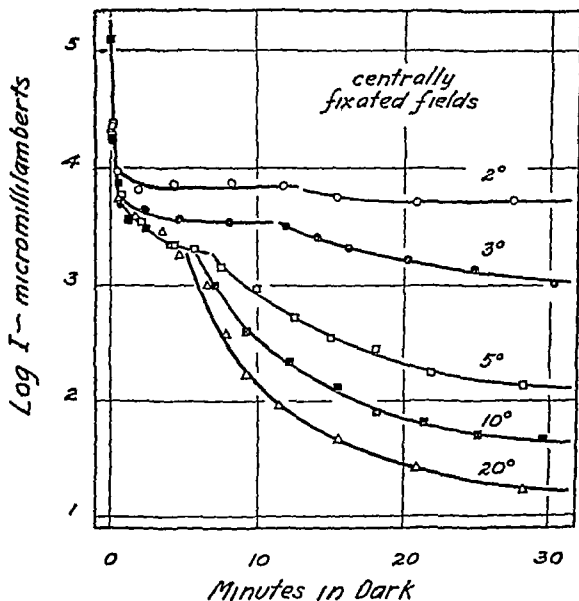


FIG 2 The threshold during dark adaptation for centrally fixated areas of different size. The primary and secondary portions of the dark adaptation curves have been separated by a slight gap since it is uncertain whether the transition between them is sharp or rounded; most likely it is rounded.

Our measurements confirm this conclusion. In the 2° field there are practically no rods, and dark adaptation is therefore mainly a foveal cone function. In a 3° area enough rods are present to show themselves when their threshold has fallen below that of the cones. In a 5° field the number of rods is of course much greater still, their

lower thresholds appear sooner, and adaptation goes lower. The same is true with the increasingly greater number of rods in the 10° and 20° fields.

It is seen in Fig 2 that the primary cone portions of the curves for areas larger than 2° are not the same as for the 2° field. This becomes understandable when it is remembered that as the area increases, there come into play not only rods but also cones outside the fovea. It is hardly to be expected that the thresholds and rates of adaptation of these peripheral cones are the same as those of the central cones.

TABLE II

*Centrally Fixated 10° Field Right Eye of C H Time in Dark in Minutes
Intensity in Micromillilamberts*

Time in dark	Log intensity
0 10	5 05
0 70	4 09
1 70	3 66
3 10	3 20
6 30	2 71
9 30	2 20
12 0	2 02
15 1	1 74
20 9	1 52
30 2	1 52
42 7	1 50
57 5	1 51
109 6	1 49
151 4	1 56
169 8	1 51

However, it is not improbable that near the transition point, especially with the larger fields the rods exert an influence on the primary threshold before they dominate in function in the secondary drop.

The data of Table I and of Fig 2 stop at about half hour of dark adaptation. The changes which take place after this time are so slight in comparison, that we have usually not continued measurements beyond 30 minutes. However, for the record, we show in Table II the average of two runs made by C H with a centrally fixated area of 10° diameter, the measurements going out to 3 hours.

It is apparent that between half hour and 3 hours in the dark the threshold does not decrease significantly

In this we can confirm Achmatov (1926) whose data show practically no threshold change in this period, and in some cases for periods lasting even 5 hours. For much more prolonged adaptation up to 24 hours Achmatov found curious, sudden additional drops in threshold separated by levels lasting several hours. The significance of these changes is hard to evaluate, it would not be surprising if they were complicated by central factors induced by lack of sleep and the prolonged stay in the dark.

TABLE III

Threshold in Log Micromillilamberts after 30 Minutes Dark Adaptation

Centrally fixated fields of different size				2° field at different distances from center			
Diameter	S.H.	G.W.	C.H.	Distance from center	S.H.	G.W.	C.H.
<i>degrees</i>				<i>degrees</i>			
1		4.03	3.45				
2	3.72	3.67	2.88	0	3.67	3.67	2.88
3	3.03	3.05	2.57				
5	2.10	2.35	1.85	2½	2.13		
10	1.64	1.73	1.55	5	1.70	1.88	1.48
20	1.20	1.30	1.20	10	1.44	1.65	1.35
				15		1.56	1.32

Our data and those of Achmatov for the threshold between half hour and 3 hours dark adaptation fail to confirm the findings reported by Kravkov and Semenovskaja (1933) and by Semenovskaja (1934). These authors find that after preliminary light adaptation to as little as 5 millilamberts and to as much as 100 millilamberts (their recorded maximum), the threshold drops as usual for about an hour but that after this it increases again. The measurements in Table II were preceded as usual by a 2 minute light adaptation to 300 millilamberts, and they show no rise in threshold during 3 hours of stay in the dark.

The most striking thing about the data for centrally located areas, presented in Table I and Fig. 2, is that as the area increases in size the intensity range covered during dark adaptation increases tremendously. The range of the 2° field between 0.2 and 30 minutes is

about 0.5 log unit whereas that for the 20° field is 3.5 log units, an increase of 1000 times. Another way of looking at this is in terms of the final threshold at about 30 minutes, which fixes the range, because the initially measured threshold immediately after light adaptation is very nearly the same for all the fields, it being probably determined by the foveal cones.

The relation between test-field diameter and final threshold is shown in Table III and in the left half of Fig. 3. The values are

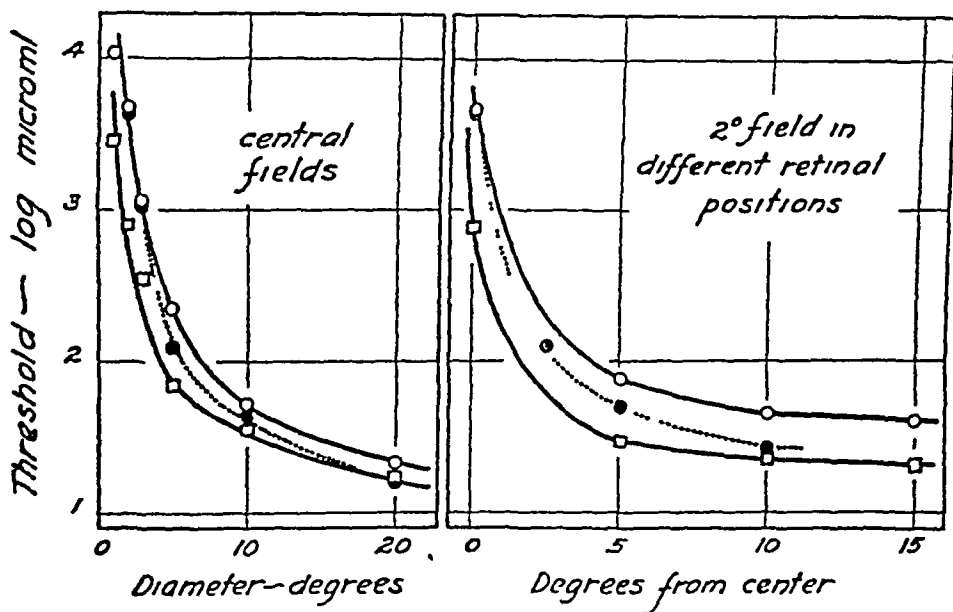


FIG. 3. A comparison of the 30 minute threshold for different sizes of central fields with the 30 minute threshold of a 2° field placed at different distances from the center of the retina.

those for 30 minutes dark adaptation and have been determined graphically from the data of Table I. The final values for the 1° central field are included here. Fig. 3 shows that the decrease in threshold with area is regular, and that no great changes are to be expected for areas beyond 20° . The basis for this regular change in threshold brings to light an important property of the retinal mosaic in relation to dark adaptation.

IV

Peripheral Fields

There exists a well established body of data describing the relation between the area of a field and the minimum intensity required to make it visible (for a summary, see Parsons, 1914) It might therefore seem that the present data of dark adaptation are merely another manifestation of this area threshold relationship, especially since the final threshold data in Table III can be approximately represented by Ricco's law (Ricco, 1877) that threshold is inversely proportional to retinal area However, it is simple to show that the major factor involved in these data of dark adaptation and final threshold has almost nothing to do with area as area, but rather with the fact that the retina is not a uniformly sensitive surface Coupled with its histological structure already referred to, it possesses a permanently graded sensitivity Experiments show that the characteristics of this gradation are the main basis for such data as shown in Figs 2 and 3

Assume for the moment that the elements in the fovea are the least sensitive ones, and that in progressing toward the periphery there appear elements which have a permanently greater and greater sensitivity to light If this were true, then the decrease in threshold with the larger, centrally fixated, retinal areas would be due to the inclusion in these fields of the permanently more sensitive elements located farther and farther away from the center

We made several sets of measurements to test this supposition The most extreme change in threshold occurs with the 20° field If the final threshold and the dark adaptation of a centrally located 20° field represent the sensitivity and behavior not of all the elements covered by this field but mainly of those elements situated most peripherally, then it should be possible to duplicate the essentials of the data by measuring the dark adaptation of the retina by means of an annulus of light covering only this peripheral region

The measurements were with a test-object composed of 1° circles situated on the circumference of a circle 20° in diameter, the appearance of the field near the threshold is that of a continuous narrow annulus of light on an unilluminated background The fixation point is in the center

The average data for three such runs with the eye of S H are given in Table IV and in Fig 4. Included in Fig 4 are the data for the 20° central field from Fig 2, and the essential, quantitative similarity of the two sets of data is obvious at a glance. The total range of the two is the same, and the final threshold is the same. The rod-cone transition is not the same in the two cases, but this is hardly to be expected since the 20° central field contains the fovea and its cones, whereas the annulus contains only peripheral cones. Moreover, the method of observation is different in the two cases: with the full field one watches for the appearance of a disc of light thus per-

TABLE IV

Annulus of 1° Circles on Circumference of a Circle of 10° Radius Right Eye of S H
Time in Dark in Minutes Intensity in Micromullilamberts

Time in dark	Log intensity
0 20	4 40
0 74	3 85
2 00	3 53
3 40	3 16
4 80	3 00
7 20	2 55
10 2	2 15
12 9	1 89
15 9	1 68
19 1	1 53
22 4	1 42
26 9	1 40

mitting the central elements considerable influence, whereas with the annulus field one is on the lookout only for the edge. As a result, in the annulus field it almost seems as if at the transition the rods and cones function together in some way to reduce the threshold,—a fact already evident even in the central field. Aside from this, however, it is plain that the main characteristics of dark adaptation as revealed by measurements with a 20°, centrally fixated field are determined by the behavior of the most sensitive elements situated in the periphery of the retinal area corresponding to the 20° field, and the size of the area as such is of secondary importance.

In order to investigate this situation in more detail, we measured dark adaptation with a 2° field placed in different positions on the retina. The data are in Table V. With G W and C H the measurements for the differently placed fields were made in separate runs. With S H all the retinal positions were tested successively in the course of one run. The measurements are the averages of two or more runs. We made similar measurements with a 1° field placed in

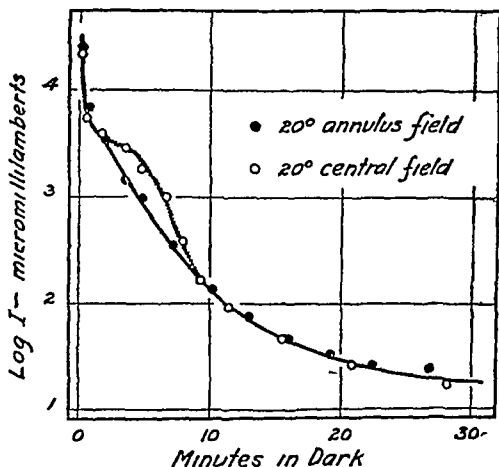


FIG. 4. Comparison between the dark adaptation of a centrally placed 20° field and that of a narrow (1°) annulus 20° in diameter.

various off center positions, the data are so similar to those in Table V that it would serve no useful purpose to print them.

The data for S H (three runs) are shown graphically in Fig. 5, which is to be compared with Fig. 2. The 2° field centrally placed is about the same in the two cases. Placed $2\frac{1}{2}^\circ$ from the center, a 2° field yields data which correspond approximately to the behavior of a 5° whole field fixated centrally. Placed 5° from the center, it

TABLE V

2° Field Different Distances from Center Time in Dark in Minutes Intensity in Micromillilamberts

Distance from center	0°		2½°		5°		10°		15°	
Observer	Time in dark	Log intensity	Time in dark	Log intensity	Time in dark	Log intensity	Time in dark	Log intensity	Time in dark	Log intensity
S H	0 22	4 40								
	0 78	3 87								
	1 70	3 66								
			5 40	3 72	4 90	3 41				
					8 50	3 27	8 30	2 80		
			11 8	3 10	13 5	2 40	14 5	2 04		
			17 8	2 64	20 0	2 09	20 9	1 65		
			25 1	2 23	26 9	1 76	28 8	1 45		
	30 9	3 67	33 1	2 09	34 7	1 63	35 5	1 41		
	38 9	4 55	41 7	2 15	43 7	1 54	45 7	1 32		
G W	52 5	4 56	55 0	1 93	55 0	1 50	56 2	1 38		
					0 26	4 37	0 30	4 40	0 55	4 42
					0 63	3 98	1 00	3 87		
					1 20	3 84			1 20	4 03
					1 80	3 77	1 90	3 60		
					2 90	3 39	2 90	3 41	2 20	3 74
					4 00	3 21	4 40	3 18	3 70	3 31
					4 90	3 11	5 40	3 01	5 00	3 14
					6 80	2 83	6 60	2 78	6 30	2 84
					8 70	2 57	8 10	2 59	8 10	2 67
					10 7	2 34	10 2	2 24	11 2	2 31
					13 5	2 20	12 3	2 05	13 2	2 09
							14 1	1 97	14 8	1 99
					16 6	2 01	16 2	1 91	18 5	1 80
					20 4	1 94	20 3	1 76	21 4	1 64
					24 0	1 93	24 0	1 71		
					27 5	1 89	28 9	1 65	26 1	1 61
C H					0 60	4 36	0 28	4 40	0 30	3 76
					1 20	3 71	1 80	3 39		
					2 30	3 34			2 10	3 23
					4 10	3 19	3 60	2 97	4 20	2 52
					5 60	3 12				
					7 20	2 69	6 90	2 64	7 40	2 17
					9 10	2 50	10 0	2 07		
					12 0	2 09			11 5	1 85
					15 9	1 80	14 5	1 75	17 0	1 65
					20 9	1 60	19 1	1 55	23 4	1 40
					28 2	1 49	25 1	1 39		

corresponds to the 10° area centrally fixated, similarly the 10° off-center field corresponds to the 20° field centrally fixated. The thresholds at 30 minutes for these 2° off-center measurements are shown in the right half of Fig 3 for comparison with the data for centrally fixated fields. Clearly the threshold alters with retinal position much as it alters with centrally fixated areas of different size.

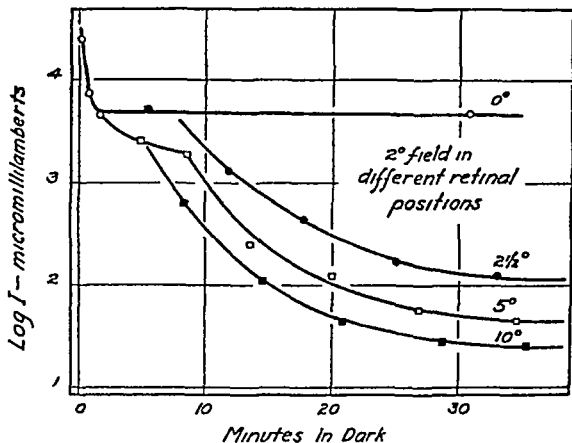


FIG 5 Dark adaptation as measured with a 2° field placed at different distances from the center. Compare this with Fig 2 for centrally fixated fields of different size.

From all this we must conclude that in centrally fixated fields of different size the general character of the dark adaptation and the value of the final threshold are determined essentially not by the area as area, but by the fact that as the areas increase in size their edges reach into regions of increasingly greater sensibility of the retina. This conclusion naturally applies only to centrally fixated areas. To study the influence on dark adaptation of area specifically as area, it will be necessary to confine the measurements to a peripheral region of the retina which is essentially homogeneous in its sensibility.

SUMMARY

The decrease in threshold shown by the eye during dark adaptation proceeds in two steps. The first is rapid, short in duration, and small in extent. The second is slow, prolonged, and large. The first is probably due to cone function, the second to rod function.

In centrally located fields the two parts of adaptation change differently with area. With small, foveal fields the first part dominates and only traces of the second part appear. As the area increases the first part changes a little, while the second part covers an increasing range of intensities and appears sooner in time.

Measurements with an annulus field covering only the circumference of a 20° circle show most of the characteristics of a 20° whole field centrally located. Similarly a 2° field located at different distances from the center shows dark adaptation characteristics essentially like those of large centrally located fields whose edges correspond to the position of the central field.

Evidently the behavior in dark adaptation of centrally located fields of different size is determined in the main not by area as area, but by the fact that the retina gradually changes in sensitivity from center to periphery, and therefore the larger the field the farther it reaches into peripheral regions of permanently greater sensibility.

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RELATION OF OXYGEN TENSION AND TEMPERATURE TO THE TIME OF REDUCTION OF CYTOCHROME

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I

Since cellular respiratory pigments have characteristic absorption spectra their function within living cells can be studied by optical methods. Thus, Keilin (1925) discovered the wide distribution of one of these pigments, cytochrome, among both plants and animals and obtained evidence of its important rôle in respiration.

Suspensions of bakers' yeast in the absence of O_2 usually exhibit four absorption bands of cytochrome at 604, 565, 549, 519 $m\mu$. Using the thoracic muscles of bees, Warburg and Negelein (1931) photographed additional absorption bands at 449, 433, and 417 $m\mu$. In the presence of O no absorption bands are generally seen. For discussions of the different views on the chemical nature of cytochrome components and their position and mode of action in cellular respiration cf. Keilin (1933), Shibata and Tamiya, (1930, 1933), and Warburg (1934).

After shaking a yeast suspension with oxygen, or after bubbling oxygen through a suspension, an easily measurable period elapses before the absorption bands of reduced cytochrome are seen in the spectroscope. Keilin (1925) called this period of time the "time of reduction of cytochrome," for convenience we shall refer to it as reduction time. The experiments here reported deal with the relation between reduction time and O_2 tension at each of six temperatures in the range of 8 to 25°C.

The results of these experiments are to be considered as preliminary.

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to more accurate determinations of reduction time by a photoelectric method. For any one set of measurements at a single O_2 pressure the results are surprisingly reliable when one considers the difficulties involved in making visual judgments of a uniform end-point in the cytochrome reduction process. For the shortest intervals (20 sec) the average deviation as per cent of the mean was 4.3 per cent, for the longest periods (180 sec) 0.4 per cent was found. However, for a series of O_2 tensions studied in the course of about 10 hours the results occasionally show somewhat greater variation. (Cf Fig 1, where in one series one point deviates from the best representative line by 6.7 per cent, in another series one point is "off" by 9.4 per cent. The majority of the points, however, deviate by less than 2 per cent.) The reliability of the visual judgments is, of course, dependent upon the maintenance of a uniform state of photic sensitivity of the observer during each series of determinations as well as throughout all the series which are to be compared. In these experiments it was found difficult to maintain such a uniform level of photic adaptation throughout an entire daily period of experimentation since the observer was frequently exposed to different levels of illumination whenever the apparatus required his attention. More accurate determinations will be attempted in the future by using photoelectric cells and thermionic amplification.

II

The smallest variation in measurements of the time for the appearance of the absorption band of cytochrome C was obtained when the oxidation of the cytochrome was brought about by vigorously shaking the yeast suspension with the gas mixture. Bubbling the gases through the suspension always gave larger average deviations. The shaking procedure was therefore used in all the experiments.

A 1 quart thermos flask was converted into a tonometer by closing the mouth of the flask with a rubber stopper bearing an observation tube of 16 ml capacity (1.7 cm internal diameter) and 2 glass tubes for admitting the gas mixtures. When the flask was turned upside down the entire observation tube was filled with the test suspension. In making a measurement of reduction time 15.1 ml of yeast suspension were vigorously shaken with the gas mixture for exactly 1 minute, the flask was then quickly turned upside down and the observation tube and about one-third of the thermos flask immersed in water contained in a rectangular museum jar. Water from a thermostat, held to within $\pm 0.1^\circ C$, was constantly circulated through the museum jar at a rate of 600 ml per minute by an air

injector device. A stop-watch was started when the flask reached the down position and was stopped when the first darkening of the spectrum was discernible at $549\text{ m}\mu$. For these preliminary experiments it was assumed that the amount of O_2 diffusing into the suspension from the gas space of the tonometer during the period of observation (3 min maximum) was negligibly small. Thus, during determinations of the reduction time the suspension was essentially confined within a closed space.

Detection of the absorption spectrum was made by aid of a Zeiss microspectroscope. The instrument was held rigidly horizontal in a line with the observation tube and the light source. Light from a 100 watt Mazda lamp was passed through an optical system consisting of a condenser lens, a heat filter, an iris diaphragm, and finally through another lens which focused a bright spot of light on the observation tube at a point 2 cm from the bottom. The voltage across the lamp was adjusted at 100 volts before each determination. Sudden changes in voltage were eliminated by running the lamp on direct current from the laboratory accumulators.

A copper shield ($7 \times 8\text{ cm}$) was fastened to the observation tube. A hole 1.5 cm in diameter, was centered at a point 2 cm from the bottom of the tube. Thus, the same region of the observation tube was illuminated each time by the same total intensity of light. The shield projected 4 cm from each side and bottom of the tube, thus protecting the observer's eyes from the bright light source.

The slit of the microspectroscope was held open at a constant width for all experiments. The width was permanently fixed in the position at which the observer could just distinguish absorption in the region $549\text{ m}\mu$ when (1) the density of the suspension was 89 c.mm. of cells per ml., (2) the cytochrome was known to be in the fully reduced state, and (3) the voltage across the lamp was set at 100 volts. The light intensity and density of the yeast suspensions were of course kept constant throughout all the experiments. Obviously, any alteration in width of the slit changes the value of the reduction time.

$\text{O}_2\text{-N}_2$ mixtures were added to the tonometer from large commercial cylinders—a separate mixture in each of six cylinders. The O_2 content of each mixture was determined by the usual gas analysis procedure¹. The gas mixture was passed through a coil of lead tubing immersed in the thermostat and then into the tonometer through a glass tube extending to within 1 cm of the bottom. It was found that by passing 6 liters of gas through the tonometer complete “washing out” of the previous mixture was obtained. For this purpose a football bladder containing 6 liters when fully inflated measured the amount of gas sent through the tonometer. Using this procedure analyses of the composition of the gas mixture in the tonometer agreed to within 0.3 per cent with the analyses of the mixture in the storage tank.

The yeast, *Saccharomyces cerevisiae*, was obtained through a local dealer from N. V. Nederlandsche Gist-en Spiritusfabriek, Delft, Holland, and was stated by the company to be “nearly a pure culture as far as this can be obtained by production

¹ I am indebted to Professor J. Barcroft for making the gas analyses.

on a large scale" A fresh lot of the yeast was obtained for each experiment A stock suspension was made up in M/15 phosphate buffer mixture of pH 7.3 and kept in an ice bath at $1-2^{\circ}\text{C}$ 1 hour before beginning the measurements of the reduction time a sample was withdrawn and sufficient dextrose to make a 1 per cent solution was added, during this period oxygen was constantly bubbled through the suspension, which was kept in the ice bath At the end of 1 hour 15.1 ml of the sample were withdrawn with a calibrated pipette and placed in the tonometer After 15 minutes had elapsed for temperature equilibrium, the gas mixture was added in the manner stated above A fresh sample of the stock suspension was withdrawn for each $\text{O}_2\text{-N}_2$ mixture

By trial it was found that 9 gm (wet weight) of yeast suspended in 100 ml of buffer mixture gave absorption bands of optimal density Samples of each stock suspension were centrifuged for 7 min at a uniform high speed in calibrated Hopkins vaccine tubes, the density was then adjusted to 89 c mm of yeast cells per ml of suspension by diluting with buffer

The temperature of the suspension in the observation tube was determined by a thermocouple The "hot" junction was permanently inserted in the bottom of the observation tube, the "cold" junction was kept in the thermostat The temperature varied $\pm 0.1^{\circ}\text{C}$ (A.D.) during observations at any one O_2 tension and sometimes as much as $\pm 0.3^{\circ}\text{C}$ during a series of $\text{O}_2\text{-N}_2$ mixtures

III

The data are presented in Fig 1, reduction time is plotted against partial pressure of O_2 (corrected for vapor pressure) A linear relation described by the equation, $y = ax - b$, was found at each of the six temperatures studied

For similar experimental conditions, Keilin (1929) found that the time (t) for the appearance of the absorption bands of cytochrome was altered when the rate of O_2 consumption (Q_{O_2}) of the same yeast suspension was experimentally modified, *e.g.* when the stores of substrate in the cell are reduced, or when the cells are held at 52°C for 1 hour All comparisons were made at the same O_2 tension and the same temperature For these experiments Keilin (1929) found that the product $Q_{\text{O}_2} \times t$ was constant His results may be described by the expression, $Q_{\text{O}_2}t = A$, where the constant A then refers to the total amount of O_2 consumed during time t

It was first assumed that A was equal to the amount of dissolved O_2 in the yeast suspension at the end of the equilibrating period, *i.e.* $A = \frac{p_2}{760} \alpha v$, where p_2 = the partial pressure of O_2 in the gas mixture, α = the absorption coefficient of O_2 in water at 760 mm Hg and at

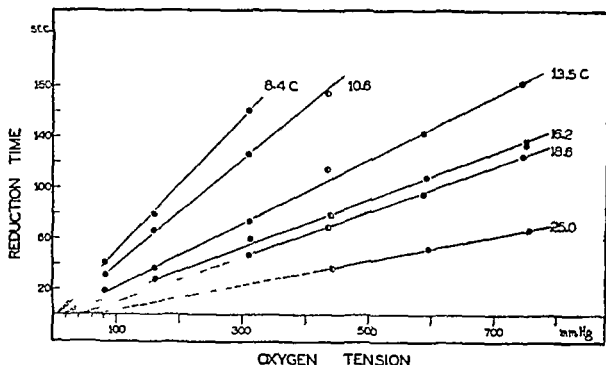


FIG 1 Relation of reduction time to O_2 tension Time for the appearance of the absorption band of cytochrome C' (ordinate) is plotted against the O_2 partial pressure (corrected for H_2O vapor tension) of the O_2 - N_2 mixture with which the yeast suspension was equilibrated Cf text for a discussion of the linear relation obtained Each point is the average of at least nine determinations of reduction time on the same sample of baker's yeast

The values of O_2 tension of the points represented by half circles were computed from a calculated value of the O_2 content of a gas mixture which escaped from its cylinder before the analysis was made For this calculation the following values were substituted in equation (2) the average reduction time obtained in experiments with the unknown gas mixture, the critical O_2 pressure for the series (p_1), and the calculated value of Q_0 for the series Since the total pressure in the tonometer was also known for each series the per cent content of O_2 could then be readily calculated The following values were obtained for the 10.6 C series, 55.0 per cent, for 18.8°C 56.8 per cent, for 16.2 C, 56.8 per cent and for 25 C, 56.2 per cent Average = 56.2 per cent

Since there are only three points in the 25 C series the x axis intercept was confirmed by calculation extrapolation gave the value 62 mm and calculation the value 63.1 mm

The x axis intercepts (p_1) were found to have the following values for the 8.4 C series, 4 mm Hg 10.6°C, 5 mm 13.5 C, 16 mm 16.2°C, 18 mm, 18.6 C, 46 mm, 25°C, 62 mm

the temperature² existing during the determinations of t , v = the total volume of yeast suspension used, 760 = normal pressure (The

² Temperature corrections were taken from the data of Bohr and Bock in Landolt, H and Börnstein R., Physikalisch-chemische Tabellen Berlin, Julius

value, 760, is inserted for convenience in calculating the value of α at p_2) However, after the results were obtained and plotted as in Fig 1, it was seen that the above expression did not hold for all conditions of temperature and O_2 pressure In Fig 1 it is seen that for each temperature the lines relating t to p when prolonged do not go through the origin, the lines intercept the x -axis yielding values (p_1) which increase with temperature The volume of O_2 remaining in solution at $p_1 = \frac{p_1}{760} \alpha v$ Therefore, at each temperature A must be equal to $\frac{p_2 - p_1}{760} \alpha v$ We may now write the general form of the equation as,

$$Q_{O_2}, t = \frac{p_2 - p_1}{760} \alpha v \quad (2)$$

By transposing Q_{O_2} in equation (2) it is seen that the time for the appearance of the absorption bands of cytochrome (reduction time, t) is inversely proportional to the rate of O_2 consumption of the yeast suspension and that t is a measure of the time required by the yeast cells to remove, through their respiratory activity, the amount of O_2 represented by the expression $\frac{p_2 - p_1}{760} \alpha v$ Since Q_{O_2} remained constant (*cf* discussion below) and p_1 remained constant during a series of O_2 tensions at constant temperature (*cf* Fig 1) it follows from equation (2) that the reduction time must increase with increasing total amounts of dissolved oxygen and the relation between reduction time and O_2 tension must therefore be linear Shibata and Tamiya (1930) reported a linear relation between t and per cent O_2 at a single temperature The equation which they give cannot be used for computations of the sort undertaken in this paper

Under our experimental conditions, where the yeast suspension is contained within a closed space during determination of reduction time, the cytochrome within the yeast cell apparently acts as a

Springer, 3rd edition, 1905, 599 Values of α may differ as much as 2 per cent depending upon the source from which they are taken The precision of our method of measuring O_2 consumption by use of equation (2) is, of course, limited by the reliability of values of α

signalling-device, absorption bands are seen when the O_2 tension is reduced below a certain pressure, p_1 . Shibata and Tamiya (1930) have already shown that at or below a certain critical oxygen pressure absorption bands of cytochrome do not disappear when the yeast suspension is shaken with gas mixtures whose O_2 content is at or below this critical pressure. They also report that the critical pressure varies with temperature: at $37^\circ C$ the absorption bands remained in the presence of 9 per cent O_2 , at $1-2^\circ C$ in the presence of 1.5 per cent O_2 . In Fig. 1 it is seen that the value of the limiting pressure (the x axis intercept, p_1) also increases directly with temperature.

Critical oxygen pressures are usually encountered in studies on the relation of O_2 tension to the rate of O_2 consumption. It has been shown for yeast and many other organisms that their rate of O_2 uptake is unaffected by tensions of O_2 above definite critical values (*cf* Tang, 1933). Below the critical pressure the rate of O_2 consumption varies rapidly with O_2 pressure, yielding a hyperbolic relation. The value of the critical pressure changes with temperature in the manner indicated above (*cf* Tang, 1933, for a review of these matters).

If p_1 is not identical with the critical O_2 pressure found in the Q_0 , O_2 -tension relation but occurs at some tension below the critical pressure, then equation (2) is not quite correct without the addition of a constant. However, p_1 cannot be very far away from the critical pressure since it is seen in Table I that the calculated values of Q_0 , obtained from equation (2) agree fairly well with manometric measurements of Q_0 : average per cent difference = 6.6. Thus, as a first approximation, equation (2) is sufficiently adequate for our purpose. This whole question will be investigated by a more accurate method.

In using equation (2) for any calculation it is, of course, important that Q_0 remains constant during determinations of reduction time at any one O_2 tension and during a series of O_2 pressures. The rate of O_2 consumption of the stock suspension was measured manometrically and was found to remain constant during the total time required to traverse each series of O_2 tensions presented in Fig. 1 (*cf* also Stier, 1932-33).

It follows from the above considerations that the time for the appearance of the absorption bands of cytochrome as determined by

the experimental procedure of Keilin (1925) and Shibata and Tamiya (1930) is not a measure of the "rate of reduction" of cytochrome, *i.e.* is not a measure of the velocity of the reaction, *oxidized cy* → *reduced cy* Keilin (1925), or *cy* O_2 → *cy* (a deoxygenation, according to Shibata and Tamiya, 1930) Measurements of the velocity of reduction are difficult since one is dealing with a reversible system where the presence of an O_2 tension above the critical pressure maintains the cytochrome in the fully oxidized form and where reduction of the oxidized cytochrome is being continually brought about by the dehydrogenase-substrate systems of the cell Determinations of velocity constants for the reduction of cytochrome *C* in the intact yeast cell are given in a preliminary report by Warburg (1934) In these experiments reoxidation of cytochrome was prevented by quickly adding KCN to the oxygenated suspension, the formation of reduced cytochrome was followed by photoelectric measurements of the density of absorption at 550 $m\mu$

IV

Calculations of the rates of O_2 consumption (Q_{O_2}) can be readily made by using equation (2) and the data presented in Fig 1 In Table I these values of Q_{O_2} are compared with those obtained by a manometric method

A further test of the reliability of the Q_{O_2} values calculated from measurements of the reduction time of cytochrome can be made by ascertaining their mathematical relationship to temperature and then comparing the constants in the equation with those found for the manometric series of Q_{O_2} determinations Choosing, for example, the Arrhenius equation,

$$k_2/k_1 = e^{\frac{\mu}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)},$$

the same values of the constant μ , should be obtained for both series

* In the Arrhenius equation, k_1 and k_2 are velocity constants (or figures proportional thereto) at the absolute temperature T_1 and T_2 , e is the base of natural logarithms, R is the gas constant, and μ is a constant with the dimensions of calories per gram molecule

of Q_{O_2} values. Unfortunately, the data are not numerous enough to make a conclusive comparison. In Fig 2, rates of O_2 uptake determined by the cytochrome method are plotted against $^{\circ}C$. The experimental data plotted in Fig 2 are fitted by the Arrhenius formula with $\mu = 19,600$, between 8.4 and $16.2^{\circ}C$ and $\mu = 13,600$ between 18.6 and $25.0^{\circ}C$ (cf legend of Fig 2 for details about the reliability

TABLE I

A Comparison between Q_{O_2} Measured Manometrically and Q_{O_2} Determined by the Cytochrome Absorption Band Technique

Temperature $^{\circ}C$	Manometric method Q_{O_2}	Cytochrome method Q_{O_2}	Difference
			<i>per cent</i>
8.4	667	690	(+) 3.4
10.6	865	833	(-) 3.7
13.5	1208	1276	(+) 5.6
16.2	1512	1639	(+) 8.4
18.6	1809	1616	(-) 10.7
25.0	2891	2869	(-) 7.7
			Av 6.6 per cent

* $Q_{O_2} =$ c. mm O_2 / 10 min / ml yeast cells

The determinations of Q_{O_2} by these two methods were made within two months of each other on the same strain of yeast. Manometric Q_{O_2} values for the lower temperatures (8 to $14^{\circ}C$) were taken from the series February 13" and for the upper temperatures (16 to $25^{\circ}C$) from the series, March 11' (cf Stier, 1932-33 Fig 2). All values of Q_{O_2} in Table I are at N.T.P.

The percentage deviation from the mean of a single determination was ± 4.5 per cent for the February 13 series and ± 5.0 per cent for the March 11 series. The average difference between the two sets of Q_{O_2} values reported in Table I is not significantly greater than the experimental error of the methods used in determining these values of Q_{O_2} . We hope to increase the accuracy of each method and then make further tests of the reliability of the cytochrome absorption band method of measuring O_2 consumption.

of these values of μ). For the same strain of yeast manometric determinations gave average values of the constant μ as follows: for the range 30 to $15^{\circ}C$, $\mu = 12,400$, 15 to $30^{\circ}C$, $\mu = 19,500$ (cf Stier, 1932-33).

The above tests of reliability of the values of Q_{O_2} calculated from measurements of the reduction time of cytochrome give additional

proof that equation (2) may be used to describe the behavior of cytochrome under our experimental conditions. Under these conditions cytochrome acts as a convenient signalling-device showing absorption bands when the O_2 tension within the suspension is reduced to, or below, certain low O_2 pressures. This property of cytochrome can be utilized for rapid determinations of the rate of O_2 consumption

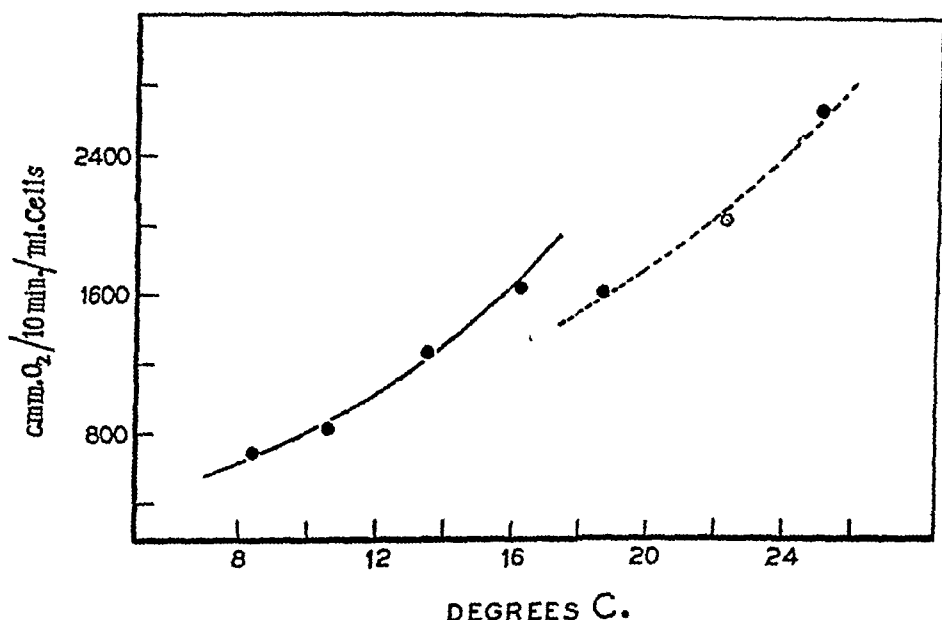


FIG. 2. Relation of temperature to rate of O_2 consumption (Q_{O_2}) calculated from measurements of reduction time of cytochrome (*cf.* text for details). The data plotted in this figure are fitted by the Arrhenius formula with $\mu = 19,600$ between 8.4 and 16.0°C and $\mu = 13,600$ between 18.6 and 25°C. The rate of O_2 consumption at 22.0°C (open circle) is an approximate value based on reduction time measurements at only two O_2 tensions. The relation between Q_{O_2} and temperature indicated by dotted lines above 16.0°C is considered an approximation since only two reliable values of Q_{O_2} were obtained in this range of temperatures.

of microorganisms containing this pigment. The accuracy of the method, in the form used in these experiments, is somewhat less than that obtained by the best manometric method. The chief difficulty, at the moment, lies in the visual method of judging the end-point of the reduction process. In future experiments it is hoped to increase both the accuracy and the speed of determining Q_{O_2} by substituting a

photoelectric detector, photographic recording, and sources of monochromatic light for the simple spectroscopic procedure employed in the experiments reported here. With these refinements it should be possible to determine rates of O_2 consumption within 1 minute, or even less. This speed of making single determinations of Q_{O_2} should be useful in the investigation of certain problems in the metabolism of microorganisms where the rate of O_2 uptake changes so rapidly with time that the slowness of the manometric method introduces large errors in the determinations of Q_{O_2} and in the analysis of the time course of the changes.

SUMMARY

The time for the appearance of the cytochrome *C* absorption band after shaking a suspension of bakers' yeast with various O_2-N_2 mixtures was determined at each of six temperatures. At each temperature a linear relation between this interval—called the reduction time—and O_2 tension was found. It was shown

- 1 That under our experimental conditions, absorption bands of cytochrome were seen when the O_2 tension of the suspension was reduced to, or below, a certain pressure which was found to be specific for each temperature (this pressure is provisionally considered to be identical with or very near to the "critical O_2 tension" usually found in $Q_{O_2}-O_2$ -tension relationships),

- 2 That the x axis intercept obtained from the *reduction time* — O_2 tension plot gives the value of the "critical" O_2 pressure at each temperature,

- 3 That the O_2 tension within the suspension is reduced by the respiratory activity of the yeast cells

An equation describing these observations is given and is used in calculating rates of O_2 consumption from measurements of reduction time of cytochrome. The average difference between the calculated values and the manometric measurements of Q_{O_2} was found to be 6.6 per cent. A rapid optical method of measuring rates of O_2 consumption based on the findings of these experiments is proposed for use with cytochrome containing microorganisms.

I wish to thank Professor Barcroft and Dr Keilin for their advice and helpfulness

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CAROTENIDS AND THE VISUAL CYCLE

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The occurrence of vitamin A in the eye tissues (Wald, 1933, 1934-35) is particularly significant because this vitamin is functionally associated with vision. Animals deprived of vitamin A become abnormally insensitive to dim light (night blind), due to failure to synthesize visual purple (Fridericia and Holm, 1925, Tansley, 1931). The present paper examines the nature of this relation and of the visual purple system.

The carotenoids of the eye tissues of several species of frog have been investigated. The pigment epithelium¹ contains large stores of vitamin A and xanthophyll esters. Light liberates from the retinal visual purple a carotenoid, retinene, which is converted by a subse

* National Research Council Fellow in Biology now at the Biological Laboratories, Harvard University. A preliminary report of this work appeared in *Nature* (1934). The *R. esculenta* measurements were made at the Institut für Physiologie of the Kaiser Wilhelm Institut für medizinische Forschung Heidelberg. I am deeply grateful to Professor Otto Meyerhof for much friendly advice and personal kindness during my stay there.

The remaining observations were made at the Physiology Laboratories of the University of Chicago. I am much indebted to Dr. Ralph W. Gerard and Dr. Anton J. Carlson for the facilities placed at my disposal there.

I am grateful to Dr. Alfred C. Redfield for critically reading the manuscript of this paper.

¹ Three distinct tissues line the fundus of the eye. Proceeding posteriorly these are the retina proper, the dark brown, single layered pigment epithelium, lying in intimate contact with the rods and cones and the black, vascular choroid. The first two are considered, for histological and physiological reasons to constitute the retina. In the present paper, the term *retina* designates the retina proper, *epithelium* denotes the pigment epithelium and this and the choroid are referred to as the *pigmented layers*.

quent thermal reaction to vitamin A. Vitamin A and retinene are also the precursors of visual purple, which they form by combining with a colloidal component, probably protein. The visual processes therefore constitute a cycle.

Franz Boll (1877) first suggested the carotenoid nature of visual purple in the paper that announced its discovery. Boll had observed that in frogs the pigment epithelium forms firm adhesions to the retina when the eye is exposed to light, implying some functional relation between this tissue and the visual process. The pigment epithelium contains golden-colored oil droplets, which fade when the eye is brightly illuminated for long periods. Visual purple, treated with dilute acetic acid, turns a yellow color which Boll believed to be identical with that of the oil droplets. He therefore proposed that the golden pigment may be the stored precursor from which visual purple decomposed by light is continuously re-synthesized.

Boll's associate Capranica (1877) identified the golden pigment with Hoppe-Seyler's "lutein" from the corpus luteum of the cow, and with the yellow pigments of egg yolk, milk, and animal fats, the group of substances now known as carotenoids. Capranica concluded "lutein" to be the parent substance of visual purple.

Kuhne and his associates discarded this hypothesis. Ewald and Kuhne (1878)² showed that visual purple and its acetic acid product possess very different properties from the epithelium pigment. Kuhne (1878)³ also found the latter substance to differ spectroscopically from the pigments of egg yolk (a mixture of xanthophyll and zeaxanthin (Kuhn and Smakula, 1931)) and of the corpus luteum of the cow (β -carotene (Kuhn and Lederer, 1931)). Many animals which possess visual purple lack entirely the epithelium pigment. In frogs its presence in the eye is of no special interest since it is distributed generally throughout the animals' fat deposits (Kuhne, 1878).⁴ The golden pigment bleaches slowly in bright light, and it is to this property that Kuhne ascribed its fading in strongly light adapted eyes (Ewald and Kuhne, 1878).² This opinion is somewhat obscured by Kuhne's additional observation (1879)⁵ that the palest epithelium droplets are found, not in the most intensely lighted areas, but just surrounding them.

These observations discredited generally Boll's hypothesis. It had been proposed without conviction since Boll remained undecided whether visual purple was a chemical substance or a physical appearance due to interference phenomena within the rods. It remains the only attempt up to the present to provide a definitive theory of the chemical nature of visual purple and the visual processes.

The identification of vitamin A in the retina revived the possibility that the visual purple system is of carotenoid nature. Haurowitz (1933) has recently

² Ewald and Kuhne, 1878, Paper II, p. 286

³ Kuhne, 1878, p. 365

⁴ Kuhne, 1878, p. 361

⁵ Kuhne, 1879, p. 310

applied carotenoid tests directly to desiccated visual purple, with negative results. Von Euler and Adler (1934) have attempted unsuccessfully to extract carotenoids from dialyzed and desiccated visual purple. These authors concluded that their experiments offer no support for the carotenoid nature of the visual pigment.

EXPERIMENTS

The frogs used in the present experiments had either been left in total darkness for at least 16 hours (dark adapted) or had been exposed to bright diffuse daylight for at least one half hour (light adapted). The former were dissected by dim red light, which does not significantly affect visual purple. Light adapted animals were dissected in daylight.

After the frogs had been beheaded and enucleated, the bulbi were opened by cutting around the rim of the sclera. The cornea and lens were lifted off, and the rear half of the eye ball was dropped into Ringer's solution. The retinas of dark adapted animals were then lifted away from the underlying tissue with a spatula, and were transferred to another container. Adhering bits of pigmented tissue were removed. The pigmented layers also were scooped out of the sclera into Ringer's solution. In light adapted frogs the pigment epithelium adheres to the retina. The basal portions of the pigmented cells may, however, be picked away with fine forceps. These contain all of the pigment epithelium with which the experiments are concerned.

After all the retinas had been prepared and cleared, the various portions of Ringer's solution were combined and centrifuged to yield the total pigmented tissue. Extractions therefore involved in every case the retina proper and the combined pigment epithelium and choroid layer.

Carotenoids of the Pigmented Layers—The pigmented tissue was washed once with distilled water to remove blood, and was extracted with chloroform or benzine. The extract is of a clear, golden color. It contains two carotenoids.

1. A golden pigment, the spectrum of which is shown in Fig. 1⁶. When dissolved in carbon disulfide it displays absorption maxima at about 445, 476, and 504 $m\mu$. In chloroform the spectrum is of similar form, but displaced so that the maxima occur at 428, 456, and 485 $m\mu$. When shaken with benzine and 90 per cent methanol, the pigment

⁶ The spectra shown in Figs. 1 and 3 were measured with the recording photoelectric spectrophotometer of Professor A. C. Hardy at the Color Measurements Laboratory of the Massachusetts Institute of Technology. This instrument has been described by Nutting (*J. Opt. Soc. America* 1934, 24, 135). The absorption is plotted as optical density or extinction $\log I_0/I$, in which I_0 is the incident and I the transmitted intensity.

enters the benzine almost quantitatively After saponification for 3 hours at room temperature in 6 per cent alcoholic KOH, this partition is reversed Pending further analysis and purification, this substance may be assumed to be an ester of xanthophyll (lutein), $C_{40}H_{64}(OH)_2$ (Kuhn and Winterstein, 1931, Kuhn and Smakula, 1931, Kuhn and Brockmann, 1932)

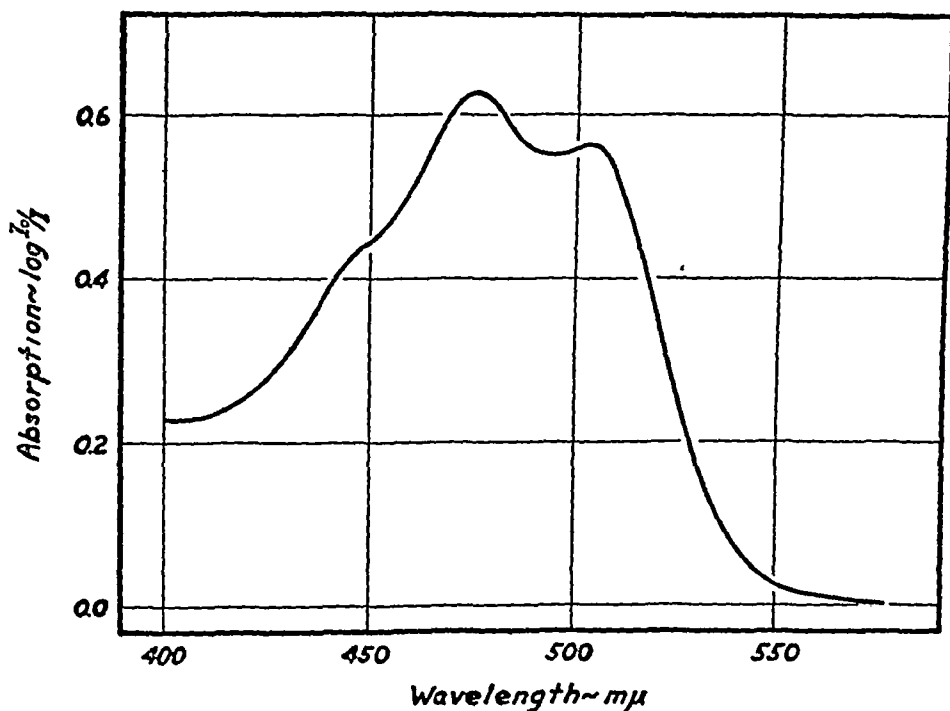


FIG 1 Absorption spectrum of an extract of pigmented layers in carbon disulfide (*R. catesbiana*) This material had been saponified and partitioned between benzine and 90 per cent methanol, the methanol fraction is shown Compare with the spectrum of crystalline xanthophyll (Kuhn and Smakula, 1931)

2 A substance which in chloroform solution possesses a single broad absorption band in the ultraviolet at 328 mμ (Fig 2) ⁷ With antimony trichloride reagent it yields a deep blue color, due to a single absorption band at about 615 mμ (crude extracts) Partitioned between benzine and 90 per cent methanol, it seeks the benzine layer,

⁷ I am indebted to Dr Elmer Miller of the Chemistry Laboratory, University of Chicago, for measuring this spectrum

and, like the xanthophyll, reverses this behavior after hydrolysis. These properties identify it as vitamin A, $C_{20}H_{30}OH$, which, judging by the partition, is present in the tissues as an ester (von Euler, Karrer, Klusmann, and Morf, 1932, Wald, 1934-35).

The concentrations of both carotenoids in the pigmented layers of light and dark adapted frogs were measured with the Pulfrich photom-

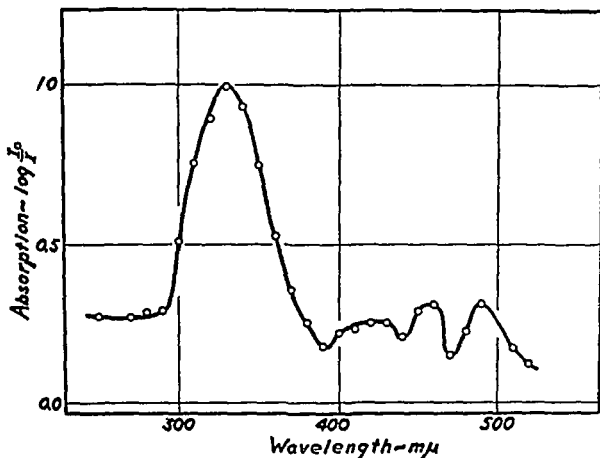


FIG. 2. Absorption spectrum of an extract of pigmented layers in chloroform (*R. pipiens*), which had been treated as in Fig. 1. The broad band at 328 mμ is due to vitamin A. Beside it is an imperfect xanthophyll spectrum, the solution having been too dilute to permit accurate measurement of this pigment.

eter (Zeiss), by methods described in the Appendix. The results are presented in Table I. The irregularity of the bull frog (*R. catesbeiana*) data is probably due to the small number of these animals used in the experiments.

In *R. pipiens* and *esculenta*, the pigmented layers of each eye contain about 4γ of vitamin A and 1γ of xanthophyll, or about 1.8 mg of the vitamin and 0.45 mg of xanthophyll per gram of dry tissue.

Light adaptation does not appreciably alter the vitamin A concentration, but causes a loss of 10 to 20 per cent of the xanthophyll. These measurements therefore confirm Boll's observation that the golden oil droplets of the pigment epithelium fade in animals exposed to bright light. The quantity of xanthophyll which disappears bears no simple relation to the duration of the exposure, for on 1 8 34 (Table I) the frogs had been exposed to daylight for 1 hour, while on 1 20 34 they had been left in daylight and bright electric light continuously for 3 days, without significant further change in xanthophyll content.

TABLE I
Carotenoids of the Pigmented Layers

Species	Dry weight per eye	Date	Number of eyes	Condition	Vitamin A per eye	Xanthophyll per eye
	mg	1933			γ	γ
<i>R. esculenta</i>	2 25	Aug 16	16	Dark adapted	4 43	0 985
		" 18	16	" "	3 92	1 00
		" 13	16	Light adapted	4 61	0 955
		" 17	16	" "	4 09	0 839
		1934				
<i>R. pipiens</i>	2	Jan 11	23	Dark adapted	3 72	1 06
		" 15	22	" "	3 47	1 15
		Feb 14	18	" "	3 65	1 17
		Jan 8	28	Light adapted	3 85	0 802
		" 20	18	" "	3 41	0 912
<i>R. catesbeiana</i>	3 5	" 25	4	Dark adapted	5 40	2 01
		Feb 19	6	" "	12 8	1 74
		Jan 22	4	Light adapted	12 7	2 67

Dark Adapted Retinas—The retinas of dark adapted frogs may be extracted thoroughly in the dark with a homopolar organic solvent like benzine or carbon disulfide without affecting the visual purple. The extracts are colorless. When highly concentrated and tested with antimony trichloride reagent, they display the vitamin A band at 615 m μ , the concentration of the vitamin is too low to appear in the present measurements.

Such retinas may be extracted subsequently with chloroform. This solvent almost immediately decolorizes the visual purple. The extract contains a greenish yellow pigment which exhibits carotenoid

properties different from any yet reported in the literature⁸ I shall refer to this substance as retinene

Retinene possesses no absorption bands in the visible spectrum Its color is due to an increasing absorption from about 500 $m\mu$ into the ultraviolet A crude extract of *R. esculenta* retinas in chloroform showed a small inflection at about 405 $m\mu$ and bands at about 310 and 280 $m\mu$, this preparation displayed a strong basic absorption so that any of these bands that may have been due to retinene had probably been displaced

Retinene reacts with antimony trichloride to yield a deep blue color, associated with a single sharp band at about 664 $m\mu$ ⁹ Carotenoids generally yield blue to green colorations with antimony trichloride, due to spectral absorptions specific for each member of the group All known natural carotenoids but vitamin A exhibit bands in this reaction which fall at 590 $m\mu$ or below (von Euler, Karrer, Klusmann, and Morf, 1932) The vitamin A band is at about 615 $m\mu$ The retinene band at 664 $m\mu$ is therefore in a wholly isolated position (Fig 3)

In the present experiments this band has been employed to identify retinene Its optical density, determined with the Pulfrich photometer, has also been used as a measure of concentration Principally, however, retinene concentrations were measured directly by the absorption of the substance in chloroform at 430 $m\mu$ The ratio between this value and the absorption of the 664 $m\mu$ band in the antimony trichloride reaction is constant, showing the yellow pigment and the substance responsible for the antimony trichloride test to be identical

Measurements of retinene concentrations in dark adapted retinas are presented in Table II These are in relative units, equal to 10 times the optical density of the chloroform solution at 430 $m\mu$, in a layer 1 cm in depth

Retinene, after extraction from the retina, is freely soluble in ben

⁸ The pigment is extracted only after shaking for some time, centrifuging, and drawing off all excess water This process is repeated several times with the same portion of chloroform Usually at about the third repetition the pigment suddenly appears in the chloroform

⁹ In the pocket spectroscope this band has invariably appeared at about 655 $m\mu$, the position previously given (Wald, 1934)

zine or carbon disulfide, yet no amount of shaking with these solvents extracts it from dark adapted retinas. It is evidently bound in such retinas within some non-lipoidal complex. The conditions of its liberation by chloroform suggest that this complex is visual purple.

Light Adapted Retinas—The retinas of frogs which have been exposed to bright daylight for one-half hour or longer are colorless, and yield with benzine or chloroform colorless extracts which contain

TABLE II
Carotenoids of the Retina

Species	Dry weight per retina	Date	Number of retinas	Condition	Vitamin A per retina	Retinene (relative units)
	mg	1933			γ	
<i>R. esculenta</i>	3	Aug. 16	16	Dark adapted	0.00	0.66
		" 18	16	" "	0.00	0.59
		" 13	16	Light adapted	0.21	0.00
		" 17	16	" "	—	0.00
		" 21	12	Bleached and faded	1.17	0.0
		1934				
<i>R. pipiens</i>	3	Jan. 11	23	Dark adapted	0.00	0.43
		" 15	22	" "	0.00	0.40
		" 8	28	Light adapted	0.34	0.00
		" 20	18	" "	0.30	0.00
		Mar. 1	36	" "	0.34	0.00
		" 8	22	Bleached, 2 min.*	—	0.27
		Feb. 14	18	" , 15 min.*	—	0.16
<i>R. catesbeiana</i>	7	Mar. 16	24	" , 60 min.*	0.81	0.00
		Jan. 25	4	Dark adapted	0.00	1.28
		" 22	4	Light adapted	1.55	0.00
		Feb. 19	6	Bleached, 45 min.*	3.61	0.00

* The times written after the notation "bleached" are periods spent at about 25°C between bleaching and extraction.

no retinene. Such extracts do contain about 0.2–0.3γ of vitamin A per *pipiens* or *esculenta* retina (Table II). The process of light adaptation which has removed visual purple and bound retinene has produced this quantity of the free vitamin.

The mechanism of these changes is revealed in experiments with isolated retinas.

The Bleaching of Visual Purple in Isolated Retinas—The retinas of dark adapted *R. pipiens*, when exposed to bright daylight, turn im-

mediately from the deep red visual purple color to bright orange (visual yellow). This fades slowly, and within about an hour at room temperature the retinas have become colorless. This sequence has been described in detail by Kühne (1878)¹⁰ and Garten (1906), and has been understood to involve purely photochemical phenomena.

It can easily be demonstrated that only the first step in the process, the conversion of visual purple to yellow, is photochemical. The subsequent decolorization of visual yellow is an ordinary thermal reaction.

If dark adapted retinas are cooled to 0°C and are exposed to bright light at this temperature, the visual purple bleaches to orange as before, but the orange color is maintained relatively unimpaired for many hours, even in bright sunlight. Upon allowing such retinas to return to room temperature, the color immediately begins to fade, and within about an hour has vanished.

Conversely, if retinas, after bleaching to orange, are placed in complete darkness at 25°C, within about an hour the visual yellow has entirely disappeared. In this case a quantity of visual purple may be regenerated, by rough estimate perhaps as much as one third the original amount. On reillumination such retinas assume a very faint orange color, due to the bleaching of the regenerated visual purple alone. This process may be repeated several times. Each time a fraction of the visual yellow reverts to purple, the remainder forming colorless products. Finally all of it has been converted to colorless material, which in the isolated retina never regenerates more than a trace of visual purple after several hours in darkness. The fading of visual yellow in retinas left continuously illuminated must similarly involve visual purple regeneration, though in this case the pigment is bleached as quickly as formed. Both situations end in colorless retinas which no longer have the power to spontaneously form appreciable quantities of visual purple.

At 0°C the regeneration of visual purple from yellow is inhibited in the same way as the formation of colorless products. Both processes have the high temperature coefficients typical of thermal reactions.¹¹

¹⁰ Kühne, 1878, p. 1

¹¹ Garten (1906) performed experiments similar to those reported in this

The appearance of visual yellow in retinas exposed to light depends upon the balance between the photochemical bleaching and thermal fading reactions. The velocity of the former process is principally controlled by the intensity of the light, that of the latter by the temperature. At room temperature and high light intensities, the yellow intermediate appears, at lower intensities decomposition to colorless products may keep pace with the bleaching process, so that the concentration of visual yellow remains inappreciable. At 0°C the removal of visual yellow is so slow that it is seen to be the primary product of bleaching over a wide range of intensities.

It is concluded that light converts visual purple in the isolated retina to visual yellow, which is removed by thermal processes in two directions: (a) reconversion to visual purple, and (b) decomposition to colorless products.

Chemistry of the Bleaching and Fading Processes—Dark adapted retinas yield retinene only after their visual purple has been destroyed with a reagent such as chloroform. After they have been exposed to light and are in the visual yellow condition, they yield their full content of retinene to benzene, carbon disulfide, or other homopolar solvents.¹² Apparently chloroform and light to this extent accomplish the same result: the disruption of visual purple and the liberation of retinene.

Visual yellow may be simply retinene. The fact that it appears greenish yellow in chloroform solution and orange in the retina does not detract from this possibility, for all of the carotenoids shift in hue from one solvent to another, and tend to assume redder tints when adsorbed. Whatever forces may hold retinene in a specific visual yellow complex must be extremely weak for so gentle a process as shaking with benzene to disrupt them. For the present, we may define retinene which cannot be extracted with benzene as bound, that which can be so extracted as free. By this criterion the effect of light upon visual purple is to liberate retinene.

section and obtained the same results. He drew from them quite different conclusions. These will be discussed critically in a subsequent paper.

¹² At the time the preliminary report of this work was written (1934) I had used only chloroform to extract bleached retinas, and so did not know of this difference between the states of retinene in them and in dark adapted retinas.

The subsequent fate of the retinene may easily be followed. Extracts of just bleached retinas contain, as do dark adapted retinas, large quantities of retinene, but no more than a trace of vitamin A. After bleached retinas have been allowed to fade somewhat at room temperature, their extracts contain smaller quantities of retinene,

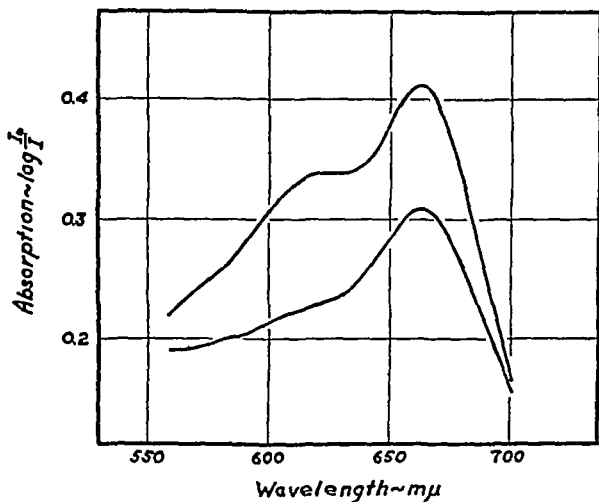


FIG 3 Absorption spectra of the antimony trichloride reaction with benzene extracts of bleached *R. catesbiana* retinas. Lower curve retinas extracted immediately upon bleaching. The test shows a strong retinene absorption at 664 $m\mu$. Upper curve retinas partly faded before extraction. The vitamin A band at 612 $m\mu$ has appeared beside the retinene absorption.

and considerable amounts of newly formed vitamin A (Fig 3). As fading proceeds, the retinene content steadily falls and that of vitamin A reciprocally rises. Finally, in wholly faded, colorless retinas, retinene has entirely vanished and vitamin A alone is found. Measurements of retinene and vitamin A concentrations made in the

course of fading are presented in Table II. The fading process is the source of the vitamin A of light adapted retinas.

The data of Table II show that the loss of about 0.63 unit of retinene from the fading *R. esculenta* retina is accompanied by the appearance of 1.17 γ of vitamin A. Similarly, about 0.42 unit of retinene in *R. pipiens* is converted into 0.81 γ of vitamin A. In both cases the ratio between the units of retinene removed and vitamin A formed is the same, about 0.5. This regularity suggests that the conversion in the isolated retina proceeds stoichiometrically, a given quantity of retinene forming its molecular equivalent of vitamin A.

It is significant that the amount of vitamin A formed in the isolated retina in this way is much greater than may be found in the retina of a light adapted animal. The essential difference between these situations is that in isolated retinas vitamin A is formed irreversibly, hence only once, while in the living animal it appears as part of a continuous process in which visual purple is resynthesized and decomposed repeatedly during light adaptation. It must be assumed that some vitamin A is lost in the visual process. Ordinarily this is replaced from sources outside the retina. The large store of vitamin A in the pigment epithelium probably serves as an immediate supply. Ultimately, however, the vitamin must be derived from the diet, and the necessity of replacing vitamin A lost during the visual cycle in this manner explains the occurrence of night blindness in animals deprived of the vitamin.

DISCUSSION

The Constitution of Visual Purple—Having concluded that visual purple contains retinene bound to some non-lipoidal material, one may inquire further into the nature of this complex.

Visual purple in bile salts solution does not diffuse through a semi-permeable membrane (Ewald and Kuhne, 1878)¹³. It can be salted out of such solutions quantitatively with magnesium or ammonium sulfate (Kuhne, 1895). These are general colloidal properties, other characteristics of visual purple suggest strongly that its colloidal residue is a protein.

In either the retina or solution, visual purple is destroyed by warm-

¹³ Ewald and Kuhne, 1878, Paper IV, p. 454.

ing to 60–70°C (Ewald and Kühne, 1878)¹⁴ The velocity of this process increases by a factor (Q_{10}) of about 7 over this range of temperatures Kühne's data for the velocity of heat destruction in fresh retinas fit the Arrhenius expression fairly well, and reveal an activation energy of about 75,000 calories per mol In acid or alkaline solution the process is accelerated and occurs at lower temperatures On the other hand, desiccated retinas are comparatively resistant to heat and require several hours to turn yellow even at 100°C

No class of phenomena now known presents exactly this combination of properties but the heat coagulation of proteins (Chick and Martin, 1910)

A second group of visual purple reactions leads to the same conclusion Acetone, alcohol, chloroform, heavy metal chlorides, and mineral acids and alkalis all quickly decolorize visual purple either in the retina or in solution (Boll, 1877, Kühne, 1879) This series of reagents possesses only one common property that of denaturing and coagulating proteins (Lloyd, 1926)¹⁵

The immediate products of visual purple decomposition by heat or chemical treatment are usually orange or yellow in color, and often these products are relatively stable to further treatment and to light This behavior, added to its other properties, links visual purple with a well defined group of chemical substances, the carotenoid proteins

Combinations of carotenoids with protein are widely distributed among animals Palmer and Eckles (1914) first discovered one in cattle serum, in which large quantities of carotene occur, bound to albumin Vegezzi (1916) and Verne (1923) have studied a number of such complexes in the decapod Crustacea Lwoff (1927) has de

¹⁴ Ewald and Kühne, 1878, Paper IV, p 440

¹⁵ Not all reagents which coagulate proteins decolorize visual purple In 4 per cent formaldehyde (Garten 1906) or alum solutions (Kühne 1878)¹⁶ its color remains intact The general coagulative changes which follow the death of the retina also do not affect it It is significant that in these cases though the photochemical bleaching process is unimpaired both the fading of visual yellow and its reversion to visual purple may be completely inhibited (Ewald and Kühne 1878)¹⁷ (Garten 1906)

¹⁶ Kühne, 1878, p 83

¹⁷ Ewald and Kühne 1878, Paper IV, p 433

scribed them in the oocytes and ocelli of a copepod, *Idya furcata* Kuhn and Lederer (1933) have investigated the constitution of such pigments from lobster shells and eggs, and have isolated their carotenoid component, astacin

All of the carotenoid-proteins possess almost identical properties, significantly parallel to those of visual purple. They are non-diffusible and may be salted from aqueous solution. They yield no color to homopolar organic solvents like benzene. They are destroyed by warming, acids, alkalis, alcohol, and acetone, usually with change in color from the purple-to-green tints of the complexes to the red-to-yellow tints of the free carotenoids which may, after such treatment, be extracted easily with the usual organic solvents. This is precisely the relation between visual purple and retinene. Among these substances, visual purple is distinguished only by its extreme light sensitivity.

A direct method of testing the protein character of visual purple might be that of enzymatic digestion. Kuhne's experiments of this nature have been inconclusive (1879)¹⁸. The digestion of whole retinas with trypsin does not affect the visual purple. However, at the close of this treatment the pigment residue is always found enclosed in keratin, which is itself not attacked by trypsin and may have protected the visual purple from the enzyme. This possibility is consistent with the observation of Ayres (1878), that a trypsin preparation which did not affect visual purple in the retina decolorized it rapidly, over a yellow intermediate, when in solution. However, Ayres found his trypsin to coagulate casein also, so that its effect upon visual purple may have been analogous to that of other protein coagulants and not a true digestive action. This type of experiment requires thorough re-investigation.

All available evidence permits the assumption that visual purple is a conjugated protein, in which retinene is the prosthetic group. Any treatment which breaks this linkage or attacks the protein may discharge the color of visual purple, and allow the yellow or orange colors due to retinene to appear.

The Precursor of Visual Purple—In order to establish the existence of a visual cycle it is necessary to show that vitamin A is the precursor

¹⁸ Kuhne, 1879, p. 267

as well as the product of the visual purple system. The present paper has so far fulfilled only the latter requirement.

It has proved impossible to synthesize visual purple from known substances *in vitro*, or even to obtain an appreciable regeneration of the pigment in isolated light adapted (colorless) retinas. One is therefore restricted to a study of the synthetic process in the intact animal, measured either by direct retinal analysis or by the determination of visual thresholds during dark adaptation, which bear a simple relation to the retinal visual purple concentration (Hecht, 1919-20, Tansley, 1931).

Studied by both these methods, visual purple regeneration in cases of vitamin A deficiency provides the most direct evidence that this vitamin is the visual purple precursor. When mammals have been deprived of vitamin A for several weeks, the synthesis of visual purple is greatly inhibited. This has been demonstrated by direct analysis in the rat (Fridericia and Holm, 1925, Tansley, 1931) and by dark adaptation studies in rats (Holm, 1925) and in man (Treitel, 1885, Kravkov and Semenovskaja, 1934). Tansley (1933) has reported that in severe avitaminosis, rat and dog retinas may form no visual purple at all. Upon re-admission of vitamin A to the diet, severe cases of night blindness may be cured with great speed (Aylroyd, 1930). Vitamin A and its carotenoid precursors are the only dietary constituents known to produce this effect.

So far as known, no vertebrate can synthesize carotenoids *de novo*. Since vitamin A is a product of visual purple decomposition, the visual pigment in turn must be derived from a carotenoid. No carotenoids occur in the frog retina but retinene and vitamin A, in the light adapted retina, only the latter. Since such retinas regenerate visual purple *in vivo* with simultaneous loss of vitamin A, it seems evident that the latter substance has been converted into the former.

The vitamin A lost in the visual process must be replaced from outside the retina. Since the frog retina contains no blood vessels (Hyrtil, 1861), all of its metabolites must enter and leave by diffusion between it and the vascular choroid, through the pigment epithelium. One might therefore expect to find the visual purple precursor in passage through these tissues. It is consistent with our argument that the pigmented layers do contain large quantities of vitamin A.

Since the frog pigment epithelium also contains xanthophyll, it might be possible that this pigment is the visual purple precursor, as originally proposed by Boll. The visual system would then act as a mechanism converting xanthophyll to vitamin A. Kuhne's criticisms of this theory, reviewed above, are still pertinent. One may add to them the following considerations: (a) Xanthophyll has not been found under any conditions in the frog retina. (b) Retinas and pigmented tissues of other animals which possess spectroscopically identical visual purple contain no xanthophyll. Such tissues do contain vitamin A (Wald, 1934-35). (c) The symptoms of vitamin A deficiency in mammals and birds are not relieved by administering xanthophyll in large quantities (Karrer, von Euler, and Rydbom, 1930, Kuhn, Brockmann, Scheunert, and Schieblisch, 1933).

It is concluded that vitamin A is the visual purple precursor, and that the visual processes are cyclic in character.

The preceding argument has rested somewhat upon information derived from experiments upon mammals, the use of which in discussing frog vision may appear questionable. Visual purple is spectroscopically identical in frogs and mammals (Kottgen and Abelsdorff, 1896). Unpublished experiments by the author have shown that vitamin A and retinene occupy the same positions in the visual purple systems of fishes and mammals as in frogs.

The fact that neither dietary night blindness nor other symptoms of vitamin A deficiency have been demonstrated in frogs, although they are frequently kept wholly without food for months at a time, presents no special difficulty. At least 3 to 6 weeks of vitamin A deprivation are required to produce marked night blindness in mammals. Frogs are usually kept in the laboratory at about 20°C below mammalian body temperature. Assuming all other conditions to be comparable, and the usual physiological temperature coefficient of about 2.5 to govern vitamin A depletion, it should take a frog about six times as long as a mammal, or from at least 4 to 8 months, to develop recognizable deficiency symptoms. This is beyond the usual survival period in the laboratory.

CONCLUSION

The results of the preceding discussion can be summarized in a diagram which may serve as a nucleus for further experiment (Fig. 4).

Most of the contents of this scheme have already been sufficiently treated

The loss of vitamin A in the visual cycle is expressed in the diagram by interpolating the term, "degradation products" This is perhaps an unfortunate name for one or more substances of which nothing is known or implied but that they are colorless vitamin A derivatives It is assumed that they eventually leave the retina by the only available route They may constitute an important functional element of the cycle, and not merely its inefficiency

Two processes have been discussed by which visual purple is synthesized in the retina reversion from visual yellow (retinene), and regeneration from colorless substances, among them vitamin A

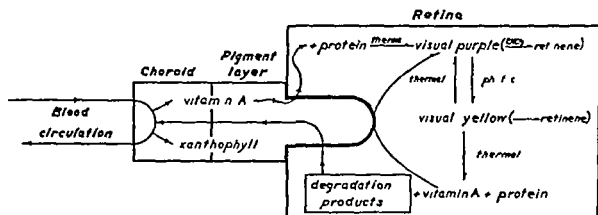


FIG. 4. Diagram of the visual purple system in the frog

These represent two distinct bases for sensory dark adaptation, and should appear in the latter function in relative amounts which vary with the extent and period of the preceding light adaptation. This possibility is now being investigated in our laboratory.

The regeneration of visual purple from yellow appears to be a simple reversal of photolysis. The synthesis from vitamin A, however, occurs only in an eye in which the relation of the retina to the pigment epithelium has remained undisturbed (Ewald and Kühne, 1878)¹⁹. The significance of this dependence is unknown. It is represented in the diagram by an arrow drawn tangent to the pigment epithelium.

The investigation of vitamin activity has heretofore been confined almost completely to the pathology of vitamin deficiency. The bril

¹⁹ Ewald and Kühne, 1878, Paper II, p. 248

liant chemical investigations of the past few years have revealed an astonishing orthodoxy in the structure of vitamins, and have provided micro-methods for identifying and measuring them in the minute concentrations in which they occur in the tissues. It has now become possible to analyze the intimate relations between vitamins and normal physiological processes. I believe the present work to be the first of such researches to yield a positive conclusion. The function of vitamin A in the visual purple cycle is that of a simple, though special, chemical component.

SUMMARY

1 Carotenoids have been identified and their quantities measured in the eyes of several frog species. The combined pigment epithelium and choroid layer of an *R. pipiens* or *esculenta* eye contain about 1γ of xanthophyll and about 4γ of vitamin A. During light adaptation the xanthophyll content falls 10 to 20 per cent.

2 Light adapted retinas contain about 0.2–0.3 γ of vitamin A alone.

3 Dark adapted retinas contain only a trace of vitamin A. The destruction of their visual purple with chloroform liberates a hitherto undescribed carotenoid, retinene. The bleaching of visual purple to visual yellow by light also liberates retinene. Free retinene is removed from the isolated retina by two thermal processes: reversion to visual purple and decomposition to colorless products, including vitamin A. This is the source of the vitamin A of the light adapted retina.

4 Isolated retinas which have been bleached and allowed to fade completely contain several times as much vitamin A as retinas from light adapted animals. The visual purple system therefore expends vitamin A and is dependent upon the diet for its replacement.

5 Visual purple behaves as a conjugated protein in which retinene is the prosthetic group.

6 Vitamin A is the precursor of visual purple as well as the product of its decomposition. The visual processes therefore constitute a cycle.

APPENDIX

Measurement of Carotenoid Concentrations

All concentrations reported in this paper were measured with the Zeiss Pulfrich photometer⁰. This instrument measures the optical density of any desired solution for narrow bands of the spectrum, isolated by monochromatic filters. Optical density is defined as $\log I_0/I$, in which I_0 is the intensity of light entering the solution, I that leaving it. For the substances and ranges of concentration with which the present work is concerned, Beer's law holds, i. e., the density is directly proportional to both the concentration and the depth of the absorbing layer. This enables one to reduce the density of a solution of any depth or concentration to a standard depth of 1 cm. and volume of 1 cc., and so permits the comparison of extracts measured under a variety of conditions.

Xanthophyll is estimated directly by determining the density of the solution in chloroform at about 470 m μ , using the S47 filter of the instrument. The density of a 1 cm. layer may be converted directly into absolute units of γ per cubic centimeter by multiplying by the factor 6.1, determined from the densities of standard solutions of crystalline xanthophyll from spinach.

Retinene is estimated similarly by the density of the chloroform solution at about 430 m μ , using filter S43. Ten times this value for a 1 cm. layer has been used as the relative measure of retinene concentration. The density of the antimony trichloride colorations of such solutions was also determined, using the S61 filter. This filter, which transmits at about 610 m μ is not well adapted for retinene determinations since the retinene antimony trichloride band maximum is at about 664 m μ . The measurements were completed within 15 seconds after mixing the reagents since the blue color which is produced in the test begins to fade immediately. The ratio between the densities obtained by direct measurement and in the antimony trichloride reaction is constant, showing the same substance to be involved in both cases.

Vitamin A concentrations were measured by the density of the band at about 615 m μ produced with antimony trichloride, using the S61 filter. The transmission of this filter coincides almost exactly with the absorption of the band and so permits a very sensitive and accurate measure of vitamin A concentration. Readings completed within 15 seconds after mixing the reagents are found to obey Beer's law. The densities may be converted to γ of vitamin per cc. of the original solution by multiplying by 34. This factor was obtained by comparing the absorption of a vitamin A solution at 328 m μ with the density at 610 m μ of the same solution in the antimony trichloride reaction. Since purified vitamin A preparations in 1 per cent solution have a density at 328 m μ of about 1300 per cm. (Heilbron, Heslop Morton, and Webster 1932) the absolute equivalent of the antimony trichloride reaction density is easily computed. The factor offered here

⁰ I wish to express my great appreciation to the firm of Carl Zeiss Inc. for the loan of a Pulfrich photometer during my stay in Chicago.

is approximate and subject to future revision. For the present purpose relative concentrations are of much greater importance than absolute, and the absolute concentrations as given are correct at least in order of magnitude.

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A FORMULATION OF THE SEROLOGICAL FLOCCULATION RATE IN THE REGION OF CONSIDERABLE ANTIBODY EXCESS

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I

Some antigen antibody reactions as they are observed in the test-tube may be divided, for convenience of study, into two phases (1) combination, and (2) the secondary aggregation which results in flocculation or agglutination. When strong concentrations of the reactants are mixed—potent hemagglutinin or precipitin and its homologous antigen—the second phase becomes immediately visible and obviously the necessarily precedent phase, combination, must have been almost instantaneous. This rapid combination cannot be explored by current immunological technic, but the second phase is susceptible of rather accurate quantitative study if suitable dilutions are used. The Ramon (constant antigen) and the Dean and Webb (constant antibody) titrations exemplify practical uses of measuring the velocity of reaction.

In a study of several simple precipitin antigen systems we have observed that in regions of considerable antibody excess the times of flocculation are a linear function of the dilutions of antigen, $1/c$, they vary inversely and in the same ratio as the change in the concentration of antigen. Similar but less regular results are recorded in several recent papers (1, 2, 7), these, however, deal mostly with complex antigens—bacteria, plasma, egg white—and because of the systems' complexity many observations are difficult or impossible to interpret with confidence. Duncan (1) records one example of linearity obtained with a polysaccharide hapten from a species of *Mycotorula*, but he comments only on the time of *optimal* flocculation as influenced

by proportional dilution of both reactants. At this equivalence point, doubling the dilution resulted in about a 2.6 fold increase of the time of particulation, i.e., the ratios are different. Jones and Little (5) found linear relationships in the *volumes* of precipitates formed by graded quantities of crystalline ovalbumin. Locke and Main (6), in a study of diphtheria toxin-antitoxin flocculation, observed a linear relationship between the L_f/L_o ratio and the log of "unit flocculation time."

TABLE I

Linear Relation of Times of Flocculation to Dilutions of Antigen

Serum, dilution	Antigen	Dilutions of antigen $\times 0.001$											
		Optimal*	1	2	7	10	15	24	41	70	120		
			Times for visible particulation in minutes										
670, 1/4 1/6	Ovalbumin	13	4	5	7	5	10	15	5	24	41	70	120
	Ovalbumin	19/5	6	5	8	5	12	17		24	42	?	100
			Dilutions of antigen $\times 0.001$										
			1	10	1	20	1	40	1	80	1	160	
775, 1/3	Edestin	5/4	8		14	5	28		56				
781, 1/3	Edestin	6			9	5	18		36	5			
	Hemocyanins												
682, 1/10	<i>L. polyphemus</i>	8	6		10		18		34	5		69	
715, 1/10	<i>B. canaliculatum</i>	2/7	4	3	8	5	16		31			64	
785, 1/20	<i>C. irroratus</i>	3/8			4	5	9		18			43	
776, 1/10	<i>Cancer sp. ?</i>	6			4	5	9	5	19	5		41	
786, 1/3	Hemoglobin	18			8		11		21			45	

* The optimal dilution is that which is equivalent to the designated dilution of serum, and is tabulated to show its distance from the zone of linearity indicated by the figures in bold faced type.

In Table I it will be seen that quite regularly, in the region where antibody concentration is 3 to 4 or more times the equivalent or optimal amount, the velocity of flocculation is very neatly a linear function of the antigen dilutions, except for very high dilutions where beginning flocculation is more difficult to detect, and where possibly the solubility of the precipitate may play a rôle, some deviations, not always in the same direction, appear.

A physicochemical interpretation of this observation may be based on von Schmöluchowski's (8) theory of the velocity of colloidal flocculation which accounts very well for figures obtained with a number of inorganic sols, using only the assumptions of the kinetic theory. He distinguishes "rapid," and "slow" flocculation. In the latter it is assumed that not all collisions of particles result in union. For this case he has

$$\Sigma = \frac{v}{1 + 4\pi R D \epsilon v t} \quad [1]$$

Where

- v_0 = the number of particles per unit volume originally
- Σ = the number of particles per unit volume after the lapse of the time, t
- D = the velocity constant of Brownian movement of the original particles
- R = the radius of the sphere of attraction of each original particle
- ϵ = the fraction of collisions resulting in union

In applying this equation to the flocculation of antigen antibody mixtures we are hampered by not knowing the magnitude of the factor ϵ , but it evidently must depend upon the amount of antibody combined with a molecule or particle of antigen. It seems reasonable to assume that with a sufficient excess of antibody a maximal change in the surface properties of the primary aggregate is produced. We suspect that this is not far from the point of maximal coating with those (most avid?) molecules of antibody which most faithfully and completely reflect the antigenic pattern.

Making this assumption it follows that the factor ϵ , whatever its value, will be constant in this region of antibody excess and may be combined with the other constants. Let $4\pi R D \epsilon = \alpha$, and expression [1] becomes

$$\Sigma = v / (1 + \alpha v t) \quad [2]$$

The degree of particulation for which we look in optimal proportion titrations is the point at which particles have just become visible to the observer under the conditions of test, or in other words, the particles have reached a certain size. Since the particles in all the tubes of a series are identical in size initially, the time required for them to reach $\beta \times$ the original size, will be the time for the total number of particles, Σ , to become equal to v / β .

Thus, if we start with tubes containing ν_a, ν_b, ν_c . particles /ml initially, the times for the number of particles to fall to $1/\beta$ of these numbers will be obtained by setting $\Sigma_v = \nu_a/\beta, \nu_b/\beta$, etc , and solving for t This gives $t_a = (\beta - 1)/\alpha\nu_a, t_b = (\beta - 1)/\alpha\nu_b$, etc or,

$$t_a : t_b : t_c \dots = 1/\nu_a : 1/\nu_b : 1/\nu_c$$

which is the proof desired

The accelerating action of shaking or partially immersing the tubes in a water bath to produce convection currents, is well known Our routine technic in the above experiments involved one-third immersion in a water bath at 37°C Freundlich (3) has derived an equation which expresses the effect of stirring on flocculations

$$\beta = \frac{4\eta r^3 N du/dz}{RT} \quad [1]$$

where β is the ratio of the number of particles which collide because of the stirring to the number which collide because of Brownian motion, as above, η is the viscosity, N is Avogadro's number, du/dz is the velocity gradient caused by the stirring, R is the gas constant, T is the absolute temperature It is seen that the effect of stirring increases as the third power of the radius of the particles Eagle (2) has pointed out certain implications of this

The chief point of interest for our purpose is that the expression of Freundlich evidently leads to the conclusion that the accelerating effect of stirring will be proportionally the same for all dilutions of antigen in this region ¹

¹ This is shown as follows Let the time for the average particle size in tube a to increase from w' to w'' be t_a' , and the time for the change from w'' to w''' be t_a'' In any other tube, b , the time required for the same changes in size w' to w'' and w'' to w''' will be t_b' and t_b'' If we make the size interval small enough, we may assume that the introduction of stirring now divides t_a' by a certain factor, c' , which from the Freundlich expression, will simply be proportional to w' Similarly t_a'' will be divided by c'' depending on t_b'' Since we are by hypothesis dealing with the same size intervals in tube b , the times t_b' and t_b'' will be divided by the same factors c' and c'' Above it was shown that $t_a/t_b = \nu_a/\nu_b$, for any

If we could evaluate ϵ , it should be possible to predict the velocity of flocculation for the entire range of antibody and antigen concentrations although it is likely that an arbitrary constant, perhaps different for each individual serum, would have to be included to allow for certain unknown and variable factors as viscosity, "avidity" etc. The most obvious assumption would be that ϵ is proportional to the fraction of the surface of each molecule of antigen which is covered by "denatured" antibody. It should be possible to check this assumption approximately and tests are being made. It would still be necessary to find an expression connecting the proportion of the surface covered with the relative amounts of antibody and antigen added to the mixture. In other words, we need two relations, [1], $Ab/An = F(AB/AN)$, where the expression Ab/An means the ratio by weight of antibody to antigen in the resulting precipitate, and AB/AN means the ratio of antibody to antigen mixed to produce this precipitate, and F , of course, is the sign of a function, and [2], $\epsilon = F'(Ab/An)$, where F' is another function. Relation [1] can be found experimentally, attempts of the authors to derive it theoretically have thus far been unsuccessful.

We have made use of the phenomenon of linearity to estimate small concentrations of antigen (*cf* Heidelberger and Kendall (4)) as in the supernatants of precipitates. If the fluid to be tested is mixed with a known excess of antibody, the time of flocculation can be used to read off the concentration of antigen, directly or by interpolation from a simple table of flocculation times, observed with the same dilution of the same antiserum under the same conditions. Considerable accuracy can be thus achieved, with the use of exceed

arbitrary interval of size. Thus $t = t_0/r$, $t_b = k t_0$. Therefore

$$\frac{\sum_{i=1}^w \frac{t}{c} + \frac{t}{c} + \frac{t}{c} + \dots}{\sum_{i=1}^w \frac{t_b}{c} + \frac{t_b}{c} + \frac{t_b}{c} + \dots} = \frac{\sum_{i=1}^w \frac{k t_0}{c} + \frac{k t_0}{c} + \frac{k t_0}{c} + \dots}{\sum_{i=1}^w \frac{t_0}{c} + \frac{t_0}{c} + \frac{t_0}{c} + \dots} = k$$

Thus it is evident that the relative times required in tubes containing different concentrations of antigen will remain the same since we are dealing with the same change in particle size in each tube.

ingly small quantities of antigens, 5 to 10 gamma, much smaller than would be required for chemical analysis or for a determination of the Dean and Webb optimum. Also, it is uncertain whether residual antibody, attached to the antigen to be estimated, might not influence the optimum, whereas in the above method any residual antibody would have no effect, as an excess sufficient to ensure maximal coating of the antigen molecules is added anyway.

SUMMARY

Attention is called to a phase of antigen-antibody reactions in which the times of flocculation are linearly proportional to the dilutions of antigen (region of considerable antibody excess), and a theoretical interpretation is offered.

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THE SOLUBILITIES OF *l* PROLINE AND *l* HYDROXYPROLINE IN WATER, THE CALCULATED HEATS OF SOLUTION, AND THE PARTIAL MOLAL VOLUME OF *l* HYDROXYPROLINE*

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The present work is a continuation of the previously reported studies on the solubilities of various amino acids in water and their thermodynamic properties (1). No precise solubility determinations on *l* proline and *l* hydroxyproline appear to have been carried out previously.

Due to the high solubility of *l* proline and *l* hydroxyproline, it was not feasible to carry out solubility determinations over as great a temperature range as in the previously reported studies. There is a tendency for these amino acids, especially hydroxyproline, to darken at higher temperatures.

It was necessary to modify somewhat the previously reported technique for the determination of solubility and partial molal volumes. A micro pipette of about 100 c mm capacity was used. It possessed a uniform capillary stem whose diameter was about 0.3 mm, 1 mm of the capillary corresponded to about 0.07 c mm. The volume of the pipette was calibrated with the aid of mercury. The amount of amino acid solution which was contained in the pipette was estimated by measuring the distance on the stem which was not filled with the solution. For the estimation of density, the pipette was filled to a certain point on the stem and the tip of the pipette was wiped with wet cotton. The pipette was left in the balance room at 25° for 15 to 30 minutes, after which it was weighed on a Kuhlmann micro balance. The capillary stem and the tip of the pipette were so

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narrow that almost no loss of solution by evaporation was observed after standing in the balance room for a 2 hour period. The amino acid content of the solution, after thoroughly rinsing the pipette, was estimated on the basis of the nitrogen content. For this purpose the micro Kjeldahl method of Parnas and Wagner (2) was used.

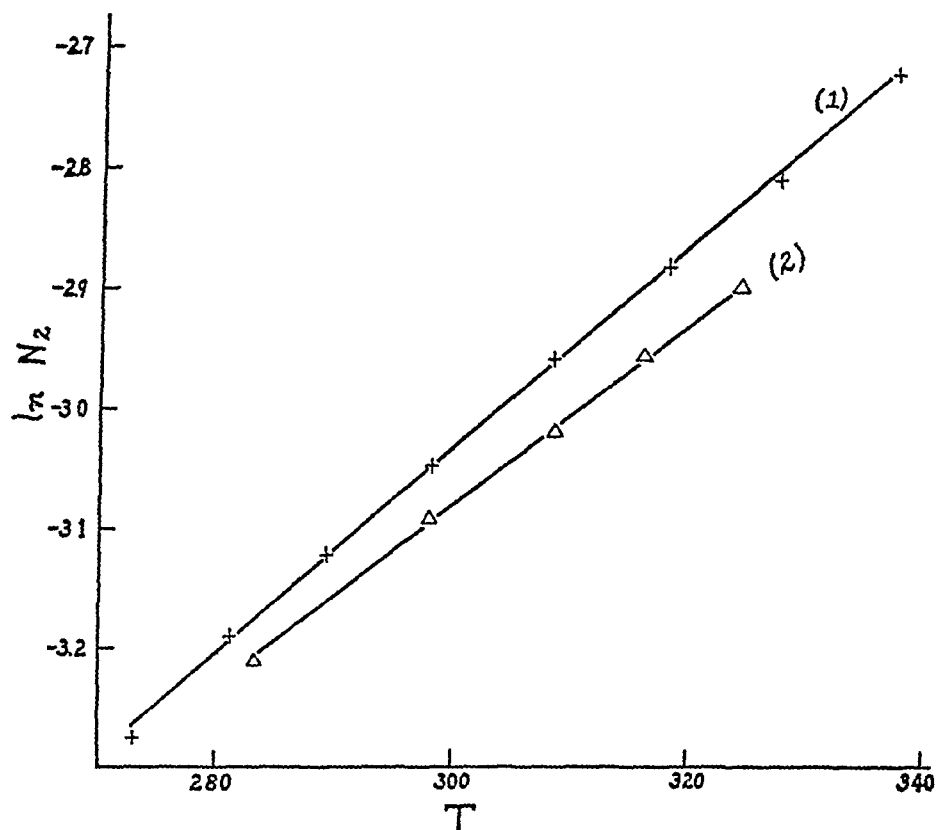


FIG. 1 Solubility of l -hydroxyproline (1) and l -proline (2). In the case of l -proline, the values of the ordinates are 1.5 less than the values indicated in the figure.

l -Proline was a commercial product. It was recrystallized 5 times from an alcohol-ether mixture. Both a commercial preparation of l -hydroxyproline and one which was prepared in this laboratory were used. They were recrystallized 3 times from 85 per cent alcohol. 14 solubility estimations of l -proline and 33 estimations of l -hydroxyproline were carried out.

The solubility-temperature relationships within the temperature

limits in which solubility estimations were carried out are represented graphically in Fig 1. The coefficients of the equations which represent these relationships, together with the calculated values for ΔH , the heat of solution, are given in Table I. The solubility equations

TABLE I
Coefficients of Solubility Equations* of *l*-Proline and *l*-Hydroxyproline

Amino acid	a_1	$b_1 \times 10^3$	a_2	a_3	$b_2 \times 10^3$	a_4	$b_4 \times 10^3$	Maximum deviation†	Mean deviation†	ΔH , cal. calculated‡
								per cent	per cent	cal
<i>l</i> -Proline	3.1050	0.4206	1.0441	-0.2407	0.9686	-3.8586	0.7586	+2.53	±1.04	1340
<i>l</i> -Hydroxyproline	2.4603	0.3891	0.3428	-1.6575	0.8959	-5.5906	0.8514	-1.53	±0.61	1506

* Solubility equations

$$\log S = a_1 + b_1 t$$

$$\log m = a_2 + b_2 t$$

$$\ln m = a_3 + b_3 T$$

$$\ln N_2 = a_4 + b_4 T$$

† Maximum deviation of the observed from the calculated values

‡ Calculated from the formula: mean deviation = $(\sum D^2/n)^{1/2}$

§ See reference (1) for method of calculation

TABLE II
Partial Molal Volumes of Solvent \bar{v}_1 and of Solute \bar{v}_2 for Solutions of *l*-Hydroxyproline at 25°

m	\bar{v}_1	\bar{v}_2
2.202	18.29	80.40
2.370	18.23	81.75
2.546	18.17	83.16
2.755	18.09	84.78
3.022	18.05	85.85
3.289	17.99	86.56
3.579	17.94	87.68

are applicable only to the temperature ranges in which solubility estimations were carried out. The reported values for ΔH are perhaps more nearly correct than those which have been reported by Zittle and Schmidt (3).

Due to the tendency of *l*-proline to crystallize out of a saturated solution, it was not feasible to carry out density determinations. The partial molal volumes for *l*-hydroxyproline only are reported. These are given in Table II. On the basis of the apparent molal volumes calculated from the empirical atomic volumes given by Traube (4), the calculated molal volume of *l*-hydroxyproline is 85.2. The validity of the calculation is based on Traube's statement, "in den ubrigen stickstoffhaltigen Ringen, wie Pyrrol, Pyrrolidin, Pyrazol, Trimethylenimid usw sind die Volumendekremente und Spannungen nicht gross."

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THE IMMUNOLOGICAL SPECIFICITY OF THE EUGLOBULIN AND PSEUDOGLOBULIN FRACTIONS OF HORSE AND HUMAN SERUM

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Serum is generally considered to contain at least two proteins, albumin and globulin, differing both in their isoelectric points, (literature summarized by Freundlich) and molecular weights (Svedberg and Sjogren), and readily separable by half saturation with ammonium sulfate, which precipitates the globulin. There has been considerable investigation tending to show that the globulin fraction itself is not homogeneous, but consists of two distinct entities. Thus we find many early investigators dividing the total globulin into water soluble and water insoluble fractions, which they called pseudoglobulin and euglobulin respectively, whereas others have applied the term euglobulin to that fraction of the serum protein precipitated by 33 per cent saturation with ammonium sulfate, and the term pseudoglobulin to the fraction precipitated between 33 and 50 per cent saturation (literature summarized by Kato).

Such differences in solubility do not, however, necessarily mean that the fractions are actually different proteins. The fact that only part of the serum globulin is precipitated on 33 per cent saturation with ammonium sulfate hardly suffices to distinguish two fractions, as even proteins known to be homogeneous can be partially precipitated by arbitrarily chosen precipitating agents. With respect to water solubility as a distinguishing criterion between protein fractions, there are reports that the water soluble fraction undergoes a progressive loss in solubility as it ages, presumably going over to euglobulin (Chuck), and that simple dialysis does not suffice to effect complete separation, which is achieved only by electrodialysis (Ruppel, Stern). Finally, Svedberg and Sjogren report that globulin

rapidly separated from serum is a homogeneous protein of uniform molecular size as determined by ultracentrifugation, but that globulin "purified" or fractionated by repeated precipitation with ammonium sulfate and dialysis has been profoundly altered, insofar as the solutions now contain particles of widely varying size

It would therefore appear that some clear-cut difference other than solubility must be found between fractions of globulin in order to establish them as distinct entities. Gay and Adler have reported that the fraction of globulin precipitated by 33 per cent saturation with ammonium sulfate sensitizes guinea pigs more readily, but causes anaphylaxis less readily, than the remainder of the globulin. Kato found the water-soluble fraction more active in both respects. Chemical differences have been reported by several investigators (Chick, Block). Hunter tested the immunological specificity of the water-soluble and water-insoluble fractions of globulin by means of the precipitin reaction. Although he was able to produce heavier precipitates in every case by adding the homologous antigen to an antiserum, there were always some demonstrable antibodies to the other fraction as well. Dale and Hartley by sensitizing guinea pigs to the globulin fractions and testing the reactivity of the excised uterus, were apparently able to demonstrate the absolute specificity of the water-insoluble fraction, but not of the water-soluble. However, numerous other investigators (Kato, Gyorffy, Otto and Iwanoff, Ruppel *et al*), have found that guinea pigs sensitized to either euglobulin or pseudoglobulin often go into anaphylactic shock on the subsequent injection of either fraction and Otto and Iwanoff have been unable to demonstrate any clear-cut difference between the two fractions using the passive anaphylaxis technique with rabbit antisera.

Not only are these results inconclusive, but even if it were possible to establish a definite immunological or chemical difference between globulin fractions, such a difference might conceivably be due to changes in the proteins induced by the physical and chemical manipulations involved in their separation, a possibility which cannot be disregarded in view of the findings of Svedberg and Sjogren already cited, and confirmed by von Mutzenbecher.

The present experiments were therefore undertaken to determine (1) whether the immunological specificity of fractions of serum globulin indicated by the anaphylaxis technique could be confirmed by a

quantitative precipitin technique, (2) if any difference which might be demonstrated was an artifact induced by the purification of the fractions, (3) to what extent lipoids associated with the several globulin fractions were responsible for their immunological specificity, as suggested by Dale and Hartley

Methods and Materials

An obvious difficulty in the work just reviewed is that even if globulin contained two or more distinct fractions, the methods of fractionation used could hardly be expected to separate them quantitatively. Each preparation would probably be a mixture of the fractions in varying proportions. In the second place, two different methods have been used to separate so called euglobulin and pseudoglobulin, and these do not yield the same products. Water soluble and water insoluble fractions each contain some material precipitable at 33 per cent saturation with ammonium sulfate and some precipitated between 33 and 50 per cent saturation, conversely, the fractions obtained at 33 and 50 per cent saturation each contain both water soluble and water insoluble protein. The serum globulin can thus actually be divided into four parts,¹ as indicated in Table I, and which of these four parts should be called euglobulin and which pseudoglobulin is a matter of arbitrary definition.

In order to minimize this confusion the two portions studied in these experiments were those satisfying both definitions: the 33 per cent precipitable water insoluble protein being arbitrarily taken as euglobulin, and the 33 to 50 per cent precipitable, water soluble protein being termed pseudoglobulin.

The experiments to be described were carried out on both human and horse serum.

Preparation of the Protein Fractions

To 150 cc. of inactivated human serum were added 75 cc. of a saturated solution of ammonium sulfate at room temperature. The resulting precipitate

¹ Strictly speaking, there are eight fractions rather than four. The four fractions obtained by first salting out with ammonium sulfate and then dialyzing (Table I) are not necessarily the same, either qualitatively or quantitatively, as those obtained by first dialyzing the serum and then further fractionating the water soluble and water insoluble proteins so obtained with ammonium sulfate.

was centrifuged, washed twice with 50 cc of 35 per cent saturated ammonium sulfate, redissolved by adding a measured minimum volume of distilled water, reprecipitated by raising the saturation with salt to 33 per cent, washed with 35 per cent salt, and finally redissolved by adding distilled water

To the supernatant from the original 33 per cent precipitate (164 cc) were added 55 cc of saturated ammonium sulfate, which made the solution 50 per cent saturated. This precipitate was now washed twice with 30 cc of a 52 per cent solution, dissolved in a measured volume of water, reprecipitated at 50 per cent, washed with a 52 per cent solution, and redissolved

The two solutions, representing the serum globulin precipitable by 33 and by 33 to 50 per cent saturation with ammonium sulfate, were dialyzed in cellophane bags against running tap water overnight, then against running distilled water overnight. The contents of each bag were centrifuged to separate the water-soluble from the water-insoluble fractions. The washed water-insoluble precipitates were dissolved in 0.85 per cent salt solution.

Portions of the 33 per cent precipitable water-insoluble (euglobulin) and of the 50 per cent precipitable water-soluble (pseudoglobulin) fractions were dehydrated from the frozen state in the Flosdorf-Mudd apparatus in order to insure the preservation of the protein, and the remainder was used for rabbit immunization.

Essentially the same method, on a somewhat larger scale, was used for the preparation of similar fractions of horse serum globulin.

Preparation of the Antisera

Nine rabbits the sera of which contained no demonstrable antibodies to human globulin were selected. Three of these received 0.5 cc of whole human serum, three received 20 mg of euglobulin in solution, and the remaining three an equal quantity of pseudoglobulin. The injections were made into the marginal ear vein three times a week for 3 weeks. At the beginning of the 4th week 30–40 cc of blood were withdrawn from the heart of each rabbit under sterile precautions, and centrifuged soon thereafter in individual bottles. The injections were continued another week and the seven surviving rabbits were again bled. There were thus obtained three different antisera against each of the three antigens in amounts varying between 30 and 70 cc. Three antisera each to whole horse serum, horse euglobulin, and pseudoglobulin—nine in all—were similarly obtained.

Nitrogen Analyses

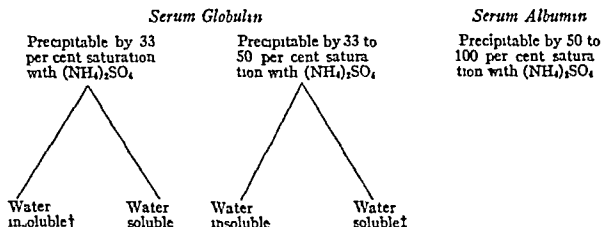
The precipitates obtained by adding antigen to antiserum as described in the text were washed in several changes of 0.85 per cent salt solution to remove excess serum and then analyzed for N content by a micro-Kjeldahl analysis, using the Meeker-Wagner technique. The results on samples containing > 1 mg N are believed to be accurate to less than 1 per cent.

Method of Extracting the Lipoids from the Globulin Fractions
(Experiment of Page 393)

In lieu of a soxhlet extraction flask a sample of each of the proteins was placed in an alundum cup which was suspended in a 150 cc flask just above 50 cc of the extracting agent (ether, petroleum ether, alcohol). A reflux condenser was fitted into the flask, so arranged that fluid dropping from the condenser would fall into the alundum cup. A water bath surrounding the flask was then heated until the rate at which the solvent dropped into the cup from the condenser just equaled the rate at which it seeped through the cup and its contents to drop into the bottom of the flask.

TABLE I

The Four Fractions of Serum Globulin Which Can Be Separated on the Basis of Salt Precipitability and Water Solubility*



* See footnote 1, page 385

† This fraction is arbitrarily taken as euglobulin throughout this paper

‡ This fraction is arbitrarily taken as pseudoglobulin throughout this paper

The extractions were maintained for 2 to 3 hours. The fluid in the bottom of the flask was then centrifuged clear of any particulate matter and evaporated. The lipid residue was taken up in a small quantity of ether, transferred to a small weighed beaker, dried, and weighed. Each sample of protein was extracted twice with anhydrous ether, and the residue then extracted twice with petroleum ether. A second sample was extracted with absolute alcohol.

The Immunological Specificity of the Fractions of Serum Globulin

If the two fractions of serum globulin chosen for study, which differ in water solubility and salt precipitability, were also entirely distinct as regards immunological properties, we would find that the antisera produced by injecting each antigen reacted specifically with

that antigen, and that only the antisera to whole serum reacted with both. Instead, it was found that every one of the three groups of antisera gave precipitation with each of the two globulin fractions, generally more marked with the homologous antigen, as Hunter has observed, but not invariably so. At first sight, this would seem to imply that the fractions were devoid of immunological specificity. However, the possibility suggested itself that there actually were two distinct globulins, but that the euglobulin and pseudoglobulin fractions as obtained by us contained both antigens in varying proportion. The following experiment was undertaken to test this possibility.

5 cc of each of the nine antisera to human serum and its fractions were placed in Wassermann tubes. Dissolved euglobulin (0.1 cc of a solution containing 19 mg/cc) was added to each of the tubes. The tubes were kept at 37°C for 3 hours (or overnight in the ice box), centrifuged, and a second portion of antigen was added. After the second or third addition excess antigen was demonstrated in the supernatant fluids by adding 0.1 cc of these to 0.2 cc of fresh rabbit anti-human serum. In each case, two further additions were made to the tubes after excess antigen in the supernatant had been thus demonstrated to assure complete precipitation of the antibody. (See Table II)²

The combined precipitates from each serum were centrifuged, washed twice in 7 to 8 cc of 0.85 per cent NaCl to remove the serum, and reserved for nitrogen analysis. 4.50 cc of each of the nine supernatant fluids which had been absorbed with euglobulin in the manner described were now treated with 1.9 mg of pseudoglobulin. In every case, a heavy precipitate formed, indicating that all the antisera contained antibodies not absorbed by euglobulin, and reacting specifically with pseudoglobulin. This residual antibody was now completely precipitated by successive additions of 1.9 mg pseudoglobulin, and the combined precipitates also washed and analyzed for nitrogen (Table II).

There were thus obtained for each serum two figures representing the amount of precipitate obtained on primary absorption with euglobulin, and the amount found when the supernatant fluid was subsequently precipitated with pseudoglobulin.

The entire procedure was now repeated in reverse order, absorbing the sera first with pseudoglobulin and then with euglobulin, with

² In spite of the excess of antigen, a slight precipitate continued to form on the addition of more antigen, insignificant in quantity as compared with the total precipitate, or that obtained on the subsequent addition of pseudoglobulin, but interesting as regards its implications (*cf* page 391).

similar results. All the sera, after complete absorption with pseudoglobulin, were found to contain residual antibodies specific for euglobulin. The results of this experiment are summarized in Table II. A similar experiment carried out with antisera to horse serum and to the globulin fractions of horse serum is summarized in Table III. The

TABLE II

The Demonstration in Antisera to Human Euglobulin, Pseudoglobulin, and Whole Serum of Antibodies Specific for the Two Globulin Fractions

Type of antiserum used	(A) Precipitate from 5 cc. antiserum on addition of euglobulin	(B) Precipitate from supernatant of (A) on addition of pseudoglobulin†	(C) Precipitate from 5 cc antiserum on addition of pseudoglobulin	(D) Precipitate from supernatant of (C) on addition of euglobulin†	Total precipitate on absorbing 5 cc. serum	
					First with euglobulin and then with pseudoglobulin (A + B)	First with pseudoglobulin and then with euglobulin (C + D)
	mg	mg	mg	mg		
Euglobulin	12.7	5.0	8.3	9.8	17.7	18.1
	28.5	2.5	17.6	8.1	31.0	25.7
	25.5	3.9	11.7	11.1	29.4	22.8
Pseudoglobulin	8.1	1.4	4.2	3.0	9.5	7.2
	3.8	0.9	1.9	2.8	4.7	4.7
	18.9	12.6	21.6	8.0	31.5	29.6
Whole serum	9.0	3.4	5.8	6.6	12.4	12.4
	14.0	8.2	11.9	7.7	22.2	19.6
	19.8	7.1	12.6	10.1	26.9	22.7

* See text for method of addition

† In Columns B and D the actual experimental readings were multiplied by 5.5/4.5 to correct for the fact that only 4.5 cc. of the supernatant were used out of a total volume of 5 + 0.5 cc. = 5.5 cc.

‡ These figures were obtained by multiplying the mg. of nitrogen in the precipitate by 6.25, assuming the protein to be 16 per cent nitrogen.

results were qualitatively similar, and the following discussion applies equally to both experiments.

As is seen in the tables, every antiserum contained fraction specific antibodies for euglobulin and pseudoglobulin. After absorbing any serum with either one of the two antigens there was always residual antibody for the other fraction as well. This result is adequately

explained on the basis that serum contains two immunologically distinct proteins which we may term globulin I and globulin II, but that the "euglobulin" and "pseudoglobulin" fractions as obtained by a combination of salting out and dialysis each contain a preponderance of one antigen and a trace of the second. On this hypothesis, euglobulin would consist largely of globulin I, but would contain a trace of globulin II, and conversely for pseudoglobulin. Since even a trace of contaminating protein would suffice to cause the formation of

TABLE III

The Demonstration in Antisera to Horse Euglobulin, Pseudoglobulin, and Whole Serum of Antibodies Specific for Two Globulin Fractions

Type of antiserum used	(A) Precipitate from 5 cc. of serum on adding euglobulin	(B) Precipitate from supernatant of (A) on adding pseudoglobulin	(C) Precipitate from 5 cc. serum on adding pseudoglobulin	(D) Precipitate from supernatant of (C) on adding euglobulin	Total precipitate on absorbing 5 cc. serum	
					First with euglobulin and then with pseudoglobulin (A - B)	First with pseudoglobulin and then with euglobulin (C - D)
Euglobulin	mg 29.2	mg 1.5	mg 9.6	mg 20.6	mg 30.7	mg 30.2
	11.0	2.1	3.1	10.6	13.1	13.7
	7.4	4.0	3.3	8.9	11.4	12.2
Pseudoglobulin	3.5	6.4	9.2	2.4	9.9	11.6
	1.1	3.5	4.1	0.1	4.6	4.2
	9.3	10.0	17.0	2.6	19.3	19.6
Whole serum	14.2	14.3	28.4	3.7	28.5	32.1
	10.3	11.8	15.7	6.6	22.1	22.3
	11.4	14.2	18.0	7.0	25.6	25.0

antibodies on injection into the experimental animals, each preparation would cause the formation of antibodies to both immunologically specific fractions. As is well known, the amounts of antibody produced to two different antigens bear no necessary relationship to the amount of antigen injected, it is nevertheless to be noted that with the exception of some of the antisera to human protein, the antisera generally contained more antibody for the homologous antigen than they did for the heterologous, corresponding to the large discrepancy in the amounts of antigen injected.

Confirmatory evidence that the foregoing is the correct explanation is furnished by two additional observations. When *e.g.* an anti-euglobulin serum was absorbed with successive small amounts of either euglobulin or pseudoglobulin, there was at first a heavy precipitate which continued to form until excess antigen was demonstrable in the supernatant fluid. Even after this excess was present, however, further additions of the antigen solution continued to cause faint precipitation. Comparatively large amounts of euglobulin failed to exhaust the antibody responsible for this slight precipitation. This phenomenon was observed with every one of the eighteen antisera, and is explained on the basis that the first additions of the euglobulin preparation removed all the antibodies for globulin I as such, but that the traces of globulin II impurity removed only a small part of the specific antibodies to that fraction. Accordingly, on further addition of euglobulin, the globulin II impurity continued to produce slight clouds of precipitate. When a solution of pseudoglobulin was now added, there was a rapid and heavy precipitation of the residual antibody to globulin II, and the supernatant then gave no precipitation with either antigen.

Another observation which bears out the thesis of incomplete separation of two immunologically distinct proteins is the fact that in every case the residual antibody to *e.g.* euglobulin after absorption of the serum with pseudoglobulin is less than the antibody precipitable by euglobulin from fresh serum. Thus, the figures in Column B are in every case less than the corresponding figures in Column C, and Column D is likewise consistently less than A. We would explain this on the basis that Column C, for example, represents the total antibody to globulin II *plus* that portion of the antibody to globulin I precipitated by the traces of the latter in pseudoglobulin, while Column B represents the total antibody to globulin II *minus* that portion precipitated by the traces of globulin II in euglobulin, and conversely for Columns A and D.

To summarize, our experimental data are adequately explained on the thesis that serum globulin consists of at least two immunologically distinct proteins, only incompletely separated even by a combination of the methods ordinarily used in separating euglobulin and pseudoglobulin.

The observation (Svedberg and Sjogren, von Mutzenbecher) that globulin repeatedly precipitated differs in such an essential property as size from the globulin in whole serum suggests that the immunological specificity of these fractions might be a similar artifact. However, the presence of these fraction-specific antibodies in the antisera to whole serum strongly suggests that this immunological difference is not an artifact due to changes in the protein induced by its physical or chemical manipulation, but is an inherent difference between two globulin fractions present as such in whole serum. It is, however, possible that in serum these two proteins are associated as a single large molecule, somehow broken up during the salting out and dialysis into its component parts.

After complete absorption of the antisera with the euglobulin and pseudoglobulin preparations, they gave no further precipitation on the addition of the "anomalous" fractions indicated in Table I: the fraction precipitated by 33 per cent saturation with ammonium sulfate, but water-soluble on subsequent dialysis, and the fraction not precipitated by the salt, but water-insoluble. It would therefore appear that the latter two globulin preparations do not contain any antigenic factor which is not also present in the euglobulin and pseudoglobulin fractions as defined in this paper.

The experimental data do not exclude the possibility that, in addition to the two globulins already demonstrated, there may be other immunologically distinct globulins which are uniformly distributed among the several fractions as obtained by salting out and dialysis, and which could therefore not be differentiated by the technique here used.

The Rôle of Lipoids Associated with the Euglobulin and Pseudoglobulin Fractions on Their Immunological Specificity

In view of the reports by Chick to the effect that euglobulin (water-insoluble globulin) can be artificially produced from pseudoglobulin (water-soluble) by the addition of a lecithin suspension to the latter, and in view also of the suggestion by Dale and Hartley that lipoid associated with euglobulin is of importance in determining its immunological properties, it became of interest to ascertain to what extent lipoids associated with the two fractions were responsible for

the specific immunological reactivity just demonstrated. Accordingly, lipoids extracted from the euglobulin and pseudoglobulin fractions were added to highly reactive antisera and the precipitate analyzed for nitrogen to determine whether the lipid particles had combined with antibody protein. As a further check, the sera so "absorbed" were tested for residual antibody to globulin, to determine whether removal of such antibodies to lipoids as might be present had in any manner affected the reactivity of the antisera to the globulin fractions.

TABLE IV

The Extraction of the Euglobulin and Pseudoglobulin Fractions of Human and Horse Serum with Ether, Petroleum Ether, and Alcohol

Extraction with	Human euglobulin			Human pseudoglobulin			Horse euglobulin			Horse pseudoglobulin		
	Sample	Lipoid extracted		Sample	Lipoid extracted		Sample	Lipoid extracted		Sample	Lipoid extracted	
		gm	per cent		gm	per cent		gm	per cent		gm	per cent
Ether	0.82	72.5	85	0.33	3.3	100	1.07	51.5	81	2.10	11.4	54
Petroleum ether		2.4	29		0.4	12		9.0	84		2.7	13
Alcohol 95 per cent	0.55	50.2	99	0.15	12.0	800	0.50	21.0	20	0.60	10.0	67
Total lipoids obtained mg		125.1			15.7			81.5			24.1	

The amounts of lipid obtained from weighed dry samples of the globulins on extraction with ether followed by petroleum ether on one sample and with 95 per cent alcohol on another are summarized in Table IV, the method of extraction being that described on page 387. These lipid fractions were pooled, brought to a concentration of 1 per cent in 95 per cent alcohol, and to this solution were added cholesterol and corn germ sterol to a concentration of 0.6 per cent of each. The alcoholic solution was now diluted by rapidly blowing in 1.3 volumes of 4 per cent NaCl, producing a microscopically crystalline suspension. The sterols were added in order to facilitate

aggregation on the addition of antisera, and to allow quantitative recovery of the lipid particles on centrifuging (Eagle). The suspensions were now analyzed for nitrogen content and added to the antisera in the quantities indicated in Table V. The lipid-antiserum mixtures were centrifuged, the precipitates washed twice to remove excess serum protein, and analyzed for nitrogen.

TABLE V

Showing That Lipids Extracted from the Globulin Fractions Do not Combine with Demonstrable Amounts of Antibodies to These Fractions

Source of lipids	Antiserum to original globulin	Lipoid suspension*	Lipoid added*	Protein from which lipid was extracted	N content of the lipid suspension	N content of sediment after centrifuging the serum lipid mixture	Antibody absorbed by the lipid	Antibody precipitable by a corresponding amount of globulin
Human euglobulin	10	5.0	21.8	143	0.05	0.03	0†	3.8
Human pseudoglobulin	10	1.0	4.4	92		Not recoverable		3.4
Horse euglobulin	10	5.0	21.8	284	0.08‡	0.11‡	0.19 (?)‡	11.2
Horse pseudoglobulin	10	1.0	4.4	380	0.06	0.04	0†	6.6

* Added in five equal parts

† The amount recovered was actually less than that added due either to incomplete recovery of the lipid particles on centrifugation or to the presence of water-soluble nitrogenous material in the lipid suspension

‡ Probably not a significant difference

In no case was there visible flocculation of the lipid particles by the antiserum, and, as seen in Table V, there was no demonstrable combination with antibody protein. In view of the fact that the antibodies in the serum used would have been completely precipitated by much less than 10 mg of globulin while the lipid extracted from 92 to 380 mg of globulin failed to combine with any demonstrable antibody we conclude that lipids extracted from the globulin frac-

tions of human and horse serum cannot combine with antibodies to these proteins

As corollaries to this finding, antisera absorbed with this large excess of lipid were not impaired either qualitatively or quantitatively in their reactivity with the globulin itself (Table VI), and globulin extracted at room temperature with ether and petroleum ether was unaffected as regards its reactivity with the antisera

TABLE VI

Showing That Antisera Absorbed with Lipoids Extracted from the Corresponding Serum Globulin Are Unaffected As to Reactivity with the Original Globulin

Antigen used for precipitation	Precipitate obtained from 5 cc. of antiserum		
	Original antiserum	Antiserum absorbed with euglobulin lipid	Antiserum absorbed with pseudoglobulin lipid
	mg	mg	mg
Human euglobulin	11.9	10.6	13.1
Human pseudoglobulin	10.6	10.0	8.1
Horse euglobulin	35.0	33.2	33.8
Horse pseudoglobulin	20.6	21.2	20.3

SUMMARY

That portion of horse and human serum globulin precipitated by 33 per cent saturation with ammonium sulfate and precipitated on subsequent dialysis was taken as euglobulin, and the fraction precipitated between 33 and 50 per cent saturation and remaining in solution on subsequent dialysis was taken as pseudoglobulin

The sera of rabbits injected with either of these antigens gave precipitation with both. However, two distinct and fraction specific antibodies could be demonstrated by absorbing the sera with the one antigen, and testing the supernatant fluid with the other. The experimental results are adequately explained on the basis that there are at least two antigenically distinct globulins in serum which we may term globulin I and globulin II and which are largely associated with so called euglobulin and pseudoglobulin respectively. The or

dinary methods of salting out and dialysis do not effect complete separation and each globulin preparation contains a trace of the other antigen. The antisera to these euglobulin and pseudoglobulin preparations therefore contain antibodies to both antigens. Each protein solution precipitates all the antibody specific for the one antigen and in addition, by virtue of the trace of contaminating protein, precipitates a *portion*, and only a portion of the antibody specific for the other antigen.

The fact that antisera to whole serum contain these same fraction-specific antibodies suggests that this immunological specificity is an inherent property of two globulins present as such in serum and is not an artifact induced by their precipitation and purification.

Lipoids extracted from the globulins by ether, petroleum ether, and alcohol give no demonstrable reaction with antisera to these globulins, antisera absorbed with a large excess of lipid are not affected as regards their reactivity with the original protein, and globulins extracted with ether and petroleum ether at room temperature are not affected as regards their reactivity with antisera. It is concluded that the immunological specificity of the globulin fractions as evidenced by the precipitation reaction is not determined by lipoids associated with the protein.

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THE KINETICS OF PENETRATION

XII HYDROGEN SULFIDE

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(Accepted for publication July 2, 1935)

INTRODUCTION

It has been shown in former papers¹ that the penetration of the weak base ammonia and the strong base guanidine into the large

CORRECTION

In Vol 19, No 1 September 20 1935 page 163 in the final portion of the equation for $\sqrt{3 \tan^{-1}}$ read $\sqrt{3 \tan^1}$

EXPERIMENTAL

The rate of entrance was studied in two ways. In one series of experiments the concentration of the sulfide ion was kept constant and the external pH varied. In the other the pH was kept constant and the concentration of sulfide ion varied.

The concentration of hydrogen sulfide in water according to some recent work by Wright and Maass,² at 25°C and a partial pressure of 755 mm is 0.1010. Since sea water has an ionic strength of about 0.7 some light acting out is to be expected. Nevertheless the solubility of the gas in sea water is sufficient to permit the use in closed systems of H₂S concentrations fairly high from the biological viewpoint, without the development of dangerous pressures or the loss of

¹ Osterhout, W. J. V. *Proc. Nat. Acad. Sci.* 1935 21, 125.

Jacques, A. G. *Proc. Nat. Acad. Sci.* 1935 21, 488.

² Wright, R. H., and Maass, O. *Canad. J. Research* 1932 6, 94.

too much gas. In order to reduce the latter as much as possible and to keep it uniform, only enough sulfide containing sea water for a single determination was made up at one time. This was mixed with the least possible agitation in a closed vessel and transferred at once to a bottle which it filled completely. The cells for experiment were added at this point and the bottle stoppered at once without gas space above the liquid. To produce uniform results it was found necessary to use cells of fairly uniform size and to stir. The stirring apparatus described in a previous paper² was used.

The pH was not determined in the sea water sample to which the cells were to be exposed as this would have involved delay and loss of gas. Instead, for each determination parallel samples were prepared, one for pH and the other for cell exposure. The pH was determined colorimetrically, using the Hellige double wedge colorimeter, the reading being referred to calibration curves as described in a previous paper⁴. After the exposure of the cells, the pH of the sea water was again determined as a check on the loss of gas. If it had changed by more than 0.15 pH unit, the run was rejected. This seldom happened.

At the outset it was our intention to use the pH determination to calculate the concentration of molecular hydrogen sulfide in the sea water from the known total sulfide concentration. However, difficulties arose which reflected seriously on the validity of these calculations and it was found that the object of the work could be attained without knowing the true pH.

After exposure the cells were washed rapidly in a stream of distilled water and dried with filter paper. The sap was then extracted by the following technique which reduces gas loss from the sap to a minimum.

Using a 1 ml "tuberculin" syringe with a fine needle, the cell was punctured and the sap was forced into the syringe, by squeezing the cell, against the opposing pressure furnished by the friction of the piston against the barrel. The sap of the first cell carried air from the needle into the syringe and this was expelled together with the sap by reversing the syringe and returning the piston to the "empty" position. This left the syringe empty but with the needle full of sap, possibly slightly deficient in H_2S , and sealed by a thin layer of liquid between the piston and barrel. Any further sap forced into the syringe was thus forced into a closed space without gas space. The sample (usually 1 ml) was measured directly in the syringe and at once introduced beneath the surface of a known quantity of a standard iodine solution. The excess of iodine was then determined by reducing it with standard thiosulfate solution in the presence of a starch indicator. Thus the amount of iodine used to oxidize the sulfide was known and from it the sulfide concentration of the sap was calculated.

As always, the question of injury remains to be considered. Observations by the eye and by feeling suggested that no injury had occurred where the exposure was less than 15 minutes. Moreover the cells not extracted, after return

⁴ Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931-32, 15, 537

to running sulfide free sea water showed no greater mortality rate than control cells not exposed to sulfide. When the exposure was for 2 hours as in the determination of equilibrium values, the unused cells exposed to the highest H_2S concentration after return to running sea water showed a slightly lower survival rate. But those which did survive appeared to be uninjured and had no more sulfate in the sap than the controls.

EXPERIMENTAL RESULTS

The rates of entrance of sulfide at the same total external sulfide concentration but at different pH (hereafter called Series 1) are given

TABLE I

Penetration into Valonia of H_2S from Sea Water at Various pH and Constant Total Sulfide Concentration

Series 1

Apparent pH Equivalent concentration at equilibrium	8.20	8.02	7.70	7.30	7.04	Unknown	Unknown
	0.0062	0.0122	0.0163	0.0246	0.032	0.0475	0.0568
Time	Equivalent concentration of sulfide in the sap						
m							
0.5		0.00118			0.00246		
1	0.00056	0.00163	0.00231	0.00313	0.00403	0.00623	0.00690
2	0.00128	0.00232	0.00387	0.00502	0.00690	0.00934	0.0126
3	0.00172	0.00336	0.00465	0.00715	0.00890	0.0131	0.0175
4	0.00195	0.00372	0.00585	0.00920	0.0106	0.0159	0.0196
5	0.00235	0.00442	0.00672	0.0112	0.0135	0.0188	0.0220
7	0.00272	0.00548	0.00785	0.0139	0.0177	0.0227	
10	0.00368	0.00368	0.00919	0.0164			

* Solutions slightly milky so that pH could not be determined

in Table I and Fig. 1. In this case as in all the other figures, the curves are drawn free hand to give an approximate fit. Where possible in the table, the "apparent" pH is given, but this, as indicated above, is probably not significant. The "equilibrium concentration" is much more important, since, as will be shown later, it is probably a fairly accurate measure of the external concentration of molecular hydrogen sulfide.

The rates of entrance where the external concentration of sulfide was varied (hereafter called Series 2) and the external pH kept constant are given in Table II and Fig. 2.

The method by which the pH was adjusted requires some comment. Throughout the experiment the same solution of 0.6 N sodium sulfide was used, and the same 0.6 N solution of hydrochloric acid was employed to adjust the pH, and

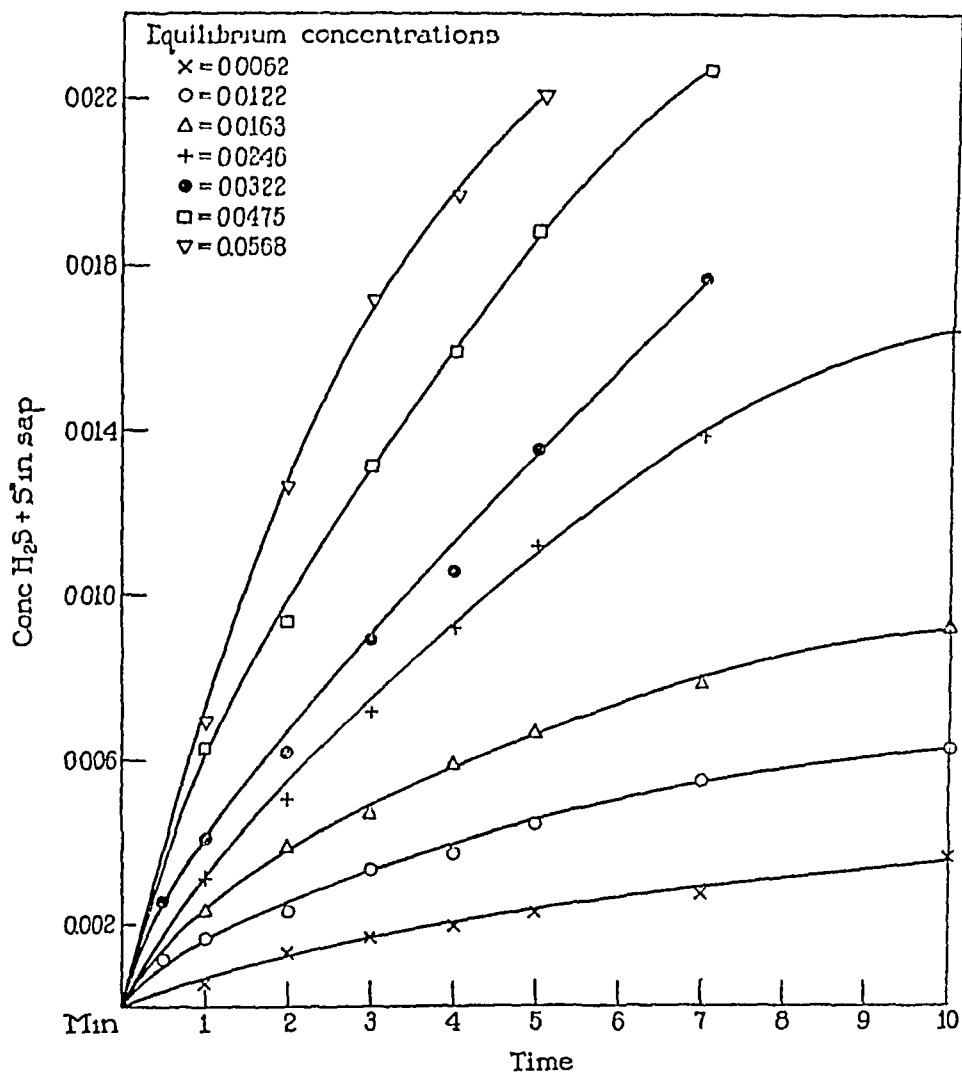


FIG. 1 Penetration of H_2S into *Valonia* from sea water containing varying concentrations of undissociated H_2S , brought about by keeping the total H_2S constant and varying the pH (Series 1)

these were added to the sea water in the same proportions. As is now generally believed, the buffer effect of normal sea water depends upon the presence of 0.002 N $NaHCO_3$ and the dissolved CO_2 which is in equilibrium with the CO_2 of

the atmosphere. It is not surprising therefore that its buffer capacity is low. In some recent measurements on sea water collected near Newport Mitchell and Rakestraw⁵ found that by the addition of 0.75 cc of 0.075 N HCl solution to 100 ml of sea water the pH was lowered from 8.2 to 7.0. The amount of acid corresponds to about 0.09 cc of 0.6 N HCl. The smallest amount of 0.6 N HCl used by us per 100 ml of sea water was 2.85 ml which was the amount required to bring the pH of a 100 ml sample of sea water 0.02 N with respect to sulfide between 7.0 to 7.1. Clearly therefore even in this case the amount of the acid used up by the ordinary buffer system of the sea water is only a fraction of that required by the new sulfide system added. Over the range of sulfide concentrations studied by us it is the added sulfide system which controls the pH and hence as long as the ratio of total sulfide to added free acid remains unchanged the pH

TABLE II
Penetration into Valonia of H₂S from Sea Water at Various Total Sulfide Concentrations and Constant pH
Series 2

Equivalent concentration in sea water Total S	0.0200	0.0500	0.0750	0.100	0.150	0.200
Time	Equivalent concentration of sulfide in the sap					
mi						
1	0.00090	0.00216	0.00320	0.00403	0.00617	0.00851
2	0.00143	0.00350	0.00537	0.00698	0.0106	0.0149
3	0.00186	0.00467	0.00746	0.00890	0.0136	0.0182
4	0.00226	0.00548	0.00883	0.0106	0.0161	0.0228
5	0.00258	0.00677	0.0115	0.0135	0.0210	0.0282
Equilibrium concentration	0.00625	0.0160	0.0250	0.0322	0.0468	0.0640

should remain constant. The apparent pH did indeed remain approximately constant, varying from 7.0 to 7.1. It should be noted that at pH 7.0 the second dissociation of H₂S is negligible since $pK_2 =$ about 15.

The uncertainty with respect to the proportion of molecular hydrogen sulfide to ionized sulfide is not quite so great in the case of the sap as in the sea water. In the first place, apparently no polysulfide (which gives the solution a faint yellow color) finds its way into the sap and no precipitate of sulfur occurs there even when the total sulfide concentration is comparatively high and the pH is far below 6.5. The uncertainty with respect to the dissociation constant persists, but by analogy with H₂CO₃ it is permissible to guess the extent of the change of pK_1 as the ionic strength increases from 0 to 0.61. According to D. A. MacInnes

⁵ Mitchell, P. H. and Rakestraw, N. W., *Biol. Bull.*, 1933, 54, 437.

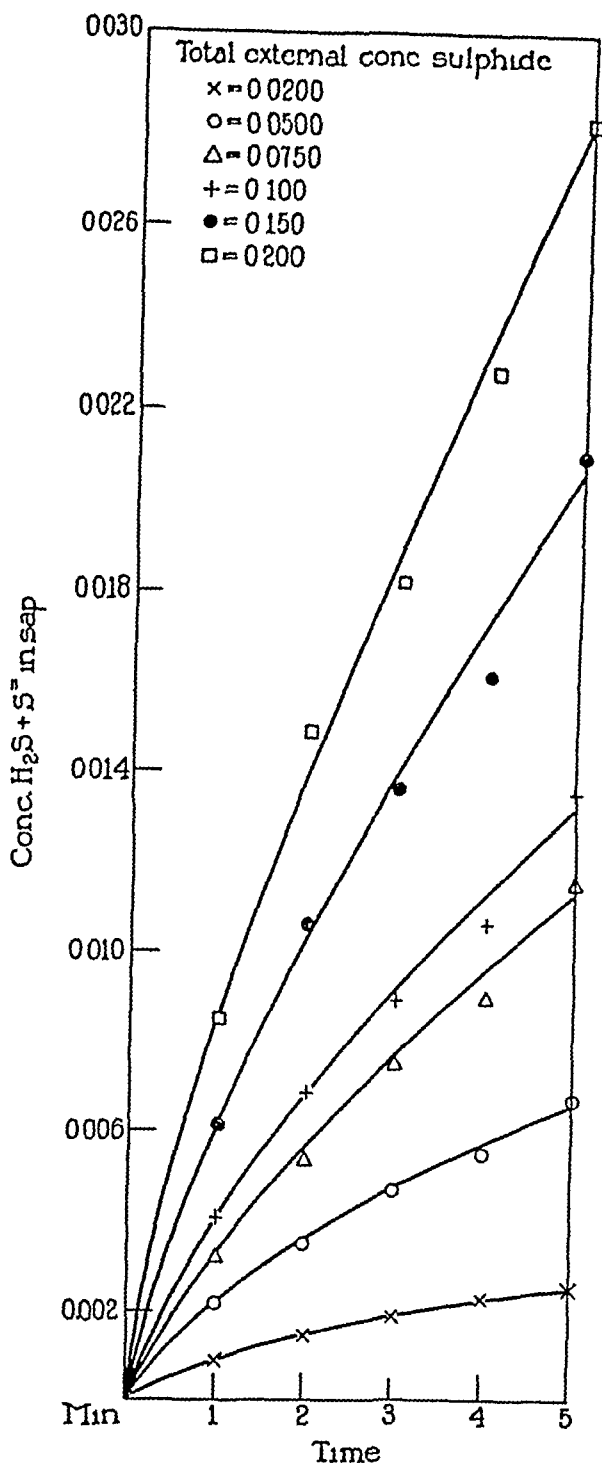


FIG 2 Penetration of H_2S into *Valonia* from sea water containing varying concentrations of undissociated H_2S , brought about by keeping the pH constant (7.0–7.1) and varying the total sulfide concentration (Series 2)

and T. Shedlovsky⁶ the limiting value for the first dissociation constant of carbonic acid pK_1 may be taken as 6.395 at 20°C. They find that this value fits in well with the recent values determined by Güntelberg and Schiödt⁷ for pA_1' at various concentrations of KCl. By interpolating from the curve of Güntelberg and Schiödt we obtain for pK_1' at 0.61 N KCl, 6.020, a shift of -0.375 unit.⁸

The pK_1 value of H₂S is not well known. According to Walker and Cormack⁹ at 18°C in 0.008 molar solution (ionic strength 0.032) pA_1' is 7.245, and according to Auerbach¹⁰ under nearly the same conditions 7.039. Since these are in relatively dilute solutions they may be provisionally taken as the limiting values at zero ionic strength. For present purposes the average 7.142 is taken. pA_1' at 0.61 ionic strength in KCl, which approximates that of the sap, is now obtained by assuming that the correction applied to H₂CO₃ may be applied here, whence $pA_1' = 7.142 - 0.375 = 6.767$.

Using this value rounded to 6.77 and the pH determination in the sap, Table III has been obtained, which shows the pH change in sap on exposure of the cells to sulfide sea water for the most dilute and most concentrated solutions in Series 2.

These results indicate that as H₂S penetrated the cell the pH in the sap decreased very rapidly to the point where the sulfide was practically all present as undissociated hydrogen sulfide.

DISCUSSION OF RESULTS

In the case of ammonia it is possible to calculate the concentration of molecular NH₃ in the sea water from the total concentration and the pH. In the present case, as pointed out above, owing to the uncertainty of the pH measurements this cannot be done. Fortunately, however, we can determine these values directly, since it seems clear that they are identical with the equilibrium concentrations.

In a former paper, Osterhout¹¹ has shown that at equilibrium¹² the

⁶ Private communication.

⁷ Güntelberg E. and Schiödt E. *Z. physik. Chem.* 1928, 135, 393.

⁸ It is worth while noting that if the value for pA_1 given by Kendall, which has hitherto been regarded as correct, i.e. 6.493, is taken the correction to ionic strength of 0.71 (that of sea water) would be about 0.48 unit. This is in good agreement with the findings of Buch and his coworkers (Buch K., Harvey H. W. and Wattenberg H. *Naturwissenschaften* 1931, 19, 773) that the decrease in the pA_1 value of H₂CO₃ in sea water is about 0.5 pH unit, pK_1 being taken as 6.472.

⁹ Walker J. and Cormack W. *J. Chem. Soc.* 1900, 77, 5.

¹⁰ Auerbach F. *Z. physik. Chem.* 1904, 49, 217.

¹¹ Osterhout W. J. V. *J. Gen. Physiol.* 1925, 8, 131.

¹² Osterhout found that it required about 1 hour to establish equilibrium, and his measurements were made after 2 hours. Our cells behaved similarly and we also allowed them to run 2 hours.

increase in the equilibrium concentration which is, as we have pointed out above, a measure of the molecular hydrogen sulfide in the sea water. Provisionally the ordinates which are concentrations may be considered as "rates," in the sense that they give the amount of sulfide

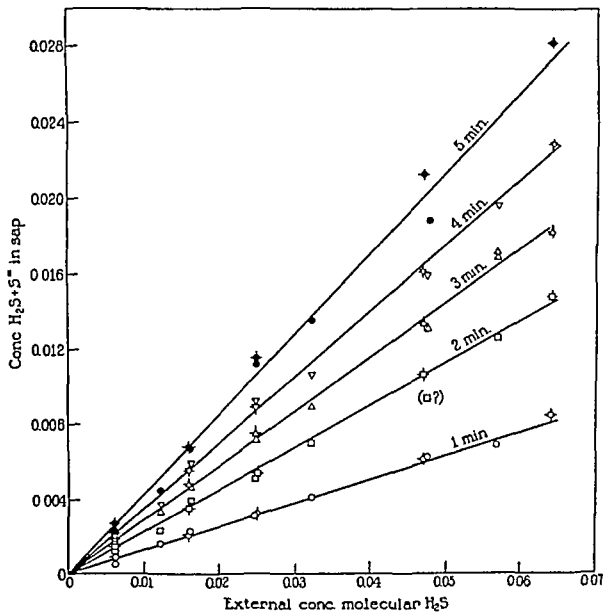


FIG. 3 Rates of entrance of H_2S into *I. alonia* at periods from 1 to 5 minutes at various external concentrations of undissociated H_2S . Plain symbols refer to Series 1 and crossed symbols to Series 2.

entering the sap when the time units are 1 to 5 minutes. The momentary rates, $\frac{d(H_2S)}{dt}$, cannot be obtained easily since the form of the rate curves is not known.

It is seen that the "rate of increase" of sulfide in the sap is directly proportional to the concentration of molecular H_2S in the sea water over the range from 1 to 5 minutes, since the curves of Fig 3 are clearly linear and pass through the origin. This behavior differs from that of ammonia or guanidine where the rate is not a linear function of the concentration of free ammonia or guanidine in the sea water ¹

In the case of ammonia, as Osterhout has shown, the form of the rate curve, x against $(\text{NH}_3)_o$, can be derived from the relationship $(\text{NH}_3)_o(\text{HX}_b - \text{NH}_4\text{X}_e) = K(\text{NH}_4\text{X}_e)$, which is the expression for the equilibrium of the reversible reaction $\text{NH}_3 + \text{HX} \rightleftharpoons \text{NH}_4\text{X}$. The subscripts o , b , and e refer respectively to the outside solution, and the beginning and the end of the reversible reaction, and ν is the total ammonia concentration in the sap, νe , ionized and unionized.

The equation gives only the relationship between the concentration of NH_4X_e and $(\text{NH}_3)_o$. But since the rate ν is assumed to be proportional to NH_4X_e we can substitute for NH_4X_e , k_1x in the equation, thus

$$(\text{NH}_3)_o(\text{HX}_b - k_1x) = k k_1 \nu$$

Putting

$$k k_1 = k_2, \quad x = \frac{(\text{NH}_3)_o \text{HX}_b}{k_2 + k_1(\text{NH}_3)_o}$$

$$\frac{\partial x}{\partial (\text{NH}_3)_o} = \frac{k_2 \text{HX}_b}{k_2 + k_1(\text{NH}_3)_o}^*$$

* k and HX_b may be regarded as constants for any one collection of cells of the same size since these will have roughly the same permeability, and the same concentration of HX in the protoplasmic surfaces. But for different collections made at different times and kept under different conditions they may be quite different.

In the case of H_2S , however, $x = k'a$ by experiment, where a is the external concentration of molecular H_2S and x is the value of $\text{H}_2\text{S} + \text{S}^-$ in the sap, and hence $\left(\frac{\partial \nu}{\partial a}\right)_t = k'$, where t signifies time. Superficially therefore it would seem that the modes of entrance for ammonia and H_2S are different.

But the question may be raised whether the H_2S curves really are linear. It might be supposed, for example, that if the external con-

centration of molecular H_2S were calculated from the pH a different set of curves would be obtained. Actually this turns out to be the case if we use the apparent pH values (Series 1) to calculate the concentration of molecular H_2S . In this case curves concave to the x axis are obtained. However, the pH values are so unreliable that no results calculated from them can be regarded as trustworthy. Fortunately the linear relationship for the H_2S curves is supported by the data of Series 2 where it is unnecessary to know the equilibrium concentration, since the sulfide sea waters were prepared in such a way as to make the pH constant while the total sulfide concentration was varied. Under these conditions the concentration of molecular hydrogen sulfide in the external solution must be directly proportional to the total sulfide concentration, so that if we plot the rates against the total sulfide concentration it would give the same form of curve, though with a different slope as the plot of rates against molecular H_2S . Such a plot is given in Fig. 4. As before, there is no question that the five curves obtained are linear.

It may be noted that the apparent pH in this series varied from 7.0 to 7.1. This must be regarded as reasonably constant for this type of experiment, but in reality it is a fairly large deviation. It is not surprising therefore that some of the points of the plot do not lie closely on the curves. But the absence of a systematic drift from the linear relationship is perfectly clear. As a further test of the linear curves of Series 1 (Fig. 3) the equilibrium concentrations for Series 2 were also obtained and the rates against these values have been plotted in Fig. 3 where they are distinguished as crossed symbols, thus \times , ϕ , etc. It will be observed that they fit smoothly onto the curves derived from Series 1.

An interesting deduction from a comparison of the two series leads to the conclusion that ionic entry plays no part in the process.

Comparing the second column of Table I with the second column of Table II we find that the rates and equilibrium concentrations were about equal. Yet in the first case the total sulfide was 0.1 N and the ionic sulfide therefore $0.1 \text{ N} - 0.0062 \text{ N} = 0.0938 \text{ N}$, while in the second case since the total sulfide was 0.02 N the ionic sulfide was $0.02 \text{ N} - 0.00625 \text{ N} = 0.01375 \text{ N}$. The rate is therefore unaffected by an almost sevenfold increase in the concentration of ionic sulfide.

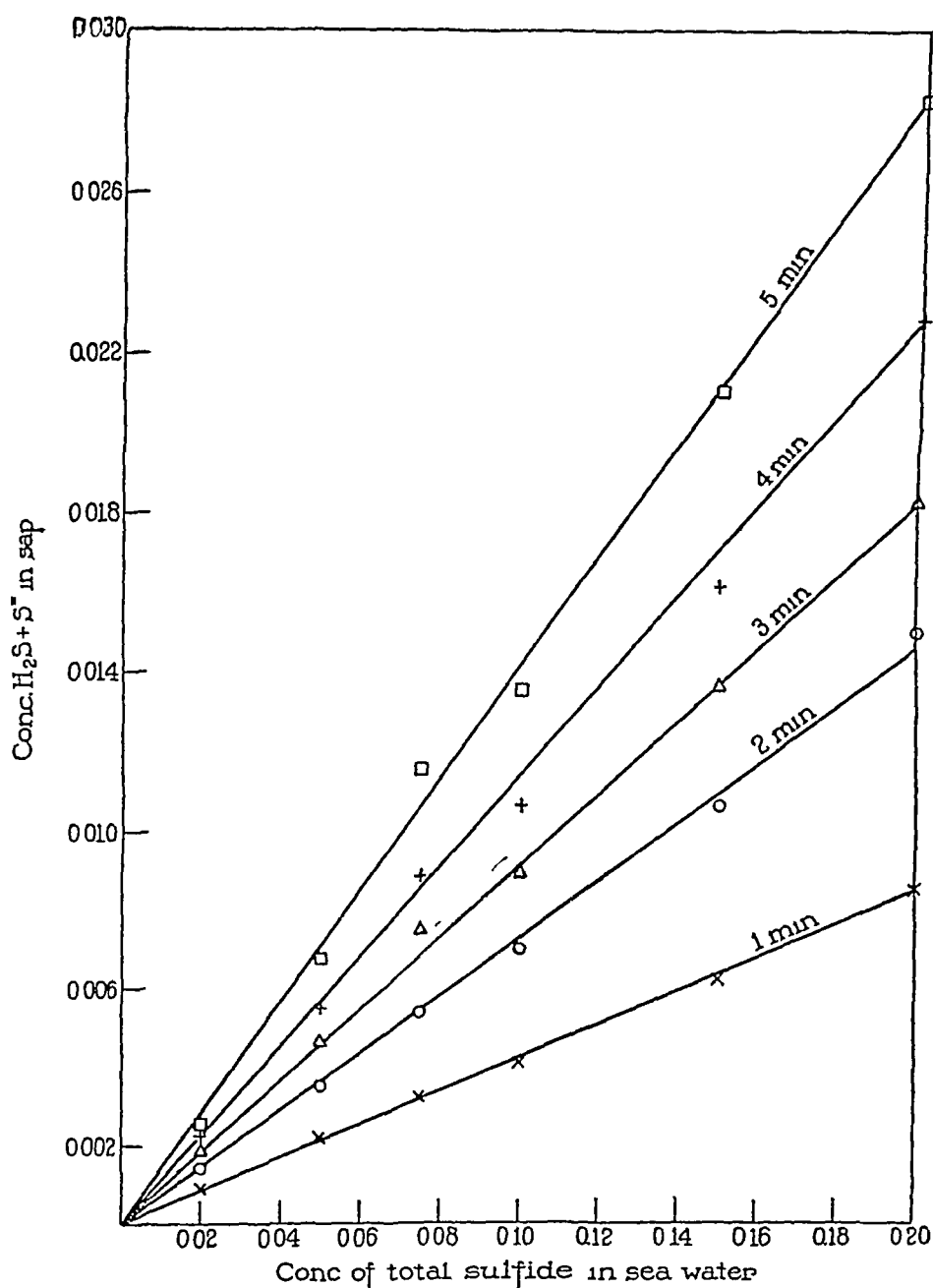


FIG 4 Rates of entrance of H_2S into *Valonia* at periods from 1 to 5 minutes at various external total sulfide ($H_2S + S^-$) concentrations pH constant (7.0 - 7.1)

The fact that H_2S curves are clearly linear and the ammonia curve clearly not, suggests that the mode of entrance is different

However, the two cases are not strictly analogous, because in the ammonia case, at the pH of the sap, a large proportion of the ammonia that enters is transformed to ammonium ion. It is true that the entrance of ammonia raises the pH. According to the results of Cooper and Osterhout,¹⁴ during the first few minutes of penetration from sea water 0.005 N with respect to NH_4Cl , it may rise from the normal value of 5.8–6.0 to 7.2. For ammonia, according to Noyes, Kato, and Sosman¹⁵ $\text{p}K_b$ at 25°C is 4.72. To get $\text{p}K'_b$ in the sap (a solution of 0.61 ionic strength the chief salt of which is KCl) we assume the same correction as was applied in the case of $\text{p}K'_1$ of H_2CO_3 , viz 0.38. $\text{p}K'_b$ is therefore $4.72 - 0.38 = 4.34$. Since at 25°C the limiting value of $K_w = 14.00$, $\text{p}K_{a2} = 9.66$.¹⁶ At $\text{pH} = 7.2$ therefore $\alpha = 99.7$ per cent and the undissociated fraction is negligible. Hence the back diffusion due to the formation of NH_4^+ at the interface between the sap and the protoplasm must be very small.

In the case of H_2S , as Table III shows, the pH of the sap falls off so rapidly that practically all the sulfide is present in the unionized form, hence the effect of this back pressure on $\left(\frac{\partial x}{\partial t}\right)_{(\text{H}_2\text{S})_o}$ and on $\left(\frac{\partial x}{\partial(\text{H}_2\text{S})_o}\right)_t$ must be taken into account.

Let us consider the general case of the penetration of a weak acid or base. Let us assume that only molecules penetrate the non aqueous surface layer of the protoplasm where they react to form a salt which passes through this layer and that in consequence the rate of passage through the protoplasm is proportional to the concentration of this salt. For the rate we can write

$$\frac{dx}{dt} = k(y - z)$$

where x is the total acid or base in the sap (ionized and unionized), y is the concentration of the salt formed at the interfaces and o and i refer to the external and internal interfaces respectively.

¹⁴ Cooper, W. C. Jr. and Osterhout, W. J. V. *J. Gen. Physiol.* 1930–31, 14, 117.

¹⁵ Noyes, A. A., Kato, Y., and Sosman, R. B., *Z. physik. Chem.*, 1910, 73, 1.

¹⁶ Note that 14.00 is the value $K_w = (\text{OH})(\text{H})$, i.e. that it is in terms of activities. The value here used is a recent one by Harned and Hawes (Harned, H. S., and Hawes, W. J. *J. Am. Chem. Soc.* 1933, 55, 2194).

According to the principles which Osterhout has applied to the ammonia case, for the layer of the protoplasm in contact with the external solution

$$a(c_o - y_o) = k_3 y_o$$

and for the layer of protoplasm in contact with the sap

$$(1 - \alpha)x(c_i - y_i) = k_3 y_i$$

where c is the concentration of base or acid in the protoplasm which reacts with the acid or base the entrance of which is being studied, α is the degree of dissociation, and a is the concentration of undissociated molecules of the penetrating acid or base which determines the reaction in the surface layer of the protoplasm. Hence

$$y_o = \frac{ac_o}{k_3 + a}$$

and

$$y_i = \frac{(1 - \alpha)x c_i}{k_3 + (1 - \alpha)x}$$

and

$$\frac{dx}{dt} = K \left(\frac{ac_o}{k_3 + a} - \frac{(1 - \alpha)x c_i}{k_3 + (1 - \alpha)x} \right)$$

For the case of ammonia the last term drops out because $1 - \alpha$ is zero or practically so.

For other cases under the conditions that $(1 - \alpha)$, c_o and c_i ¹⁷ remain constant the above expression can be integrated for a constant value of a to give the relationship between t and x ,¹⁸

¹⁷ In the case of H_2S , for example, $(1 - \alpha)$ becomes almost at once nearly unity because of the decrease in the internal pH. c_o and c_i may also be regarded as constant and equal to each other in the thin unstirred layers in which the reaction occurs.

¹⁸ The steps in integration are as follows. Collect constants, to get

$$\frac{dx}{dt} = \left(A - \frac{BX}{1 + CX} \right)$$

in which

$$A = \frac{Kac_o}{k_3 + a}, \quad B = \frac{K(1 - \alpha)c_i}{k_3}, \quad C = \frac{(1 - \alpha)}{k_3}$$

Then

$$\frac{dx}{dt} = \frac{A + ACX - BX}{1 + CX} = \frac{A + B'X}{1 + CX}$$

(Footnote continued on following page)

$$\begin{aligned}
t = & \frac{1}{C \left(\frac{K}{k_1 + a} \right) - B} \log \left[\frac{K}{k_1 + a} + \left(\frac{CK}{k_1 + a} \right) - B \right] x \\
& + \frac{C}{\left(C \frac{K}{k_1 + a} - B \right)^2} \left[\frac{K}{k_1 + a} + \left(C \frac{K}{k_1 + a} - B \right) x \right] \\
& - \frac{K}{k_1 + a} \log \left[\frac{K}{k_1 + a} + \left(\frac{CK}{k_1 + a} - B \right) x \right] \\
& - \frac{1}{C \left(\frac{K_{\infty}}{k_1 + a} - B \right)} \log A - \frac{C}{\left(C \frac{K}{k_1 + a} - B \right)^2} \left[\frac{K}{k_1 + a} \right. \\
& \left. - \frac{K_{\infty}}{k_1 + a} \log \frac{K}{k_1 + a} \right]
\end{aligned}$$

(Footnote 18 continued from preceding page)

where $AC - B = B$, or

$$\begin{aligned}
\frac{dt}{dx} &= \frac{1 + CX}{A + BX} \quad \frac{dx}{A + B\lambda} + \frac{cx dx}{A + B\lambda} \\
\int dt &= \int \frac{dx}{A + B\lambda} + C \int \frac{\lambda dx}{A + B\lambda} + I
\end{aligned}$$

or

$$t = \frac{1}{B} \log (A + BX) + \frac{C}{(B)^2} [A + B\lambda - A \log (A + B\lambda)] + I$$

when $t = 0$ $x = 0$ hence $I = -\frac{1}{B} \log A - \frac{CA}{(B)^2} (1 - \log A)$ when

$$\begin{aligned}
t = & \frac{1}{B} \log (A + B\lambda)X + \frac{C}{(B)^2} [A + B\lambda - A \log (A + B\lambda)] \\
& - \frac{1}{B} \log A - \frac{C}{(B)^2} (A - 1 \log A)
\end{aligned}$$

Substituting in the expression for B we get

$$\begin{aligned}
t = & \frac{1}{AC - B} \log [A + (AC - B)\lambda] + \frac{C}{(AC - B)^2} [1 + (AC - B)\lambda - \\
& A \log (A + (AC - B)\lambda)] - \frac{C}{AC - B} \log A - \frac{C}{(AC - B)^2} (A - A \log 1)
\end{aligned}$$

Substituting for A which contains a we get the expression above

In this expression $C = \frac{(1 - \alpha)}{k_1}$ and $B = \frac{K(1 - \alpha)c_1}{k_3}$ ¹⁹

This shows that if a (p 406) is the external concentration of molecular H_2S and x is the value of $H_2S + S^-$ inside, the value of $\left(\frac{\partial x}{\partial a}\right)_t$ is not constant when the method of entrance involves reversible chemical reactions between H_2S and a constituent of the protoplasm. Suppose, however, the acid or base enters by diffusing in the non-aqueous layers of the protoplasm in undissociated form. We can apply to this case the ideas already developed by Osterhout²⁰ and by Longworth²¹ for cell models with the guaiacol-*p*-cresol mixture as the non-aqueous phase.

Then we can write

$$\frac{dx}{dt} \sim (y_0 - y_1)$$

where y is the concentration of the diffusing molecules in the thin unstirred layer immediately in contact with the sap and the external solution respectively,

$$y_0 = S_0 a \quad \text{and} \quad y_1 = S_1(1 - \alpha)x$$

where S is the partition coefficient of the molecule between water and the non-aqueous protoplasm. Whence

$$\frac{dx}{dt} = k_4[S_0 a - S_1(1 - \alpha)x]$$

When $1 - \alpha$ is constant the expression can be integrated with a constant to give

$$\int dt = k_4 \int \frac{dx}{S_0 a - S_1(1 - \alpha)x}$$

or

$$t = \frac{1}{S_1(1 - \alpha)k_4} - \log [S_0 a - S_1(1 - \alpha)x] + I$$

¹⁹ The derivative $\left(\frac{\partial x}{\partial a}\right)_t$ could, of course, be obtained and the values of B and C substituted. This would serve no useful purpose, however, and will be omitted.

²⁰ Osterhout, W. J. V., *J. Gen. Physiol.*, 1932-33, 16, 529.

²¹ Longworth, L. G., *J. Gen. Physiol.*, 1933-34, 17, 211.

and evaluating I and putting $\frac{1}{S_i(1-\alpha)k_s} = k_s$

$$t = \frac{1}{k_s} \log \frac{S_o a}{S a - S_i(1-\alpha)x}$$

or

$$x = \frac{S_o a}{S(1-\alpha)} (1 - e^{-k_s t})$$

And if $S_o = S$ ²² as may well be the case and $1 - \alpha = 1$ as seems to be the case with H_2S , the integral becomes

$$tk_s = \log \frac{a}{a-x}$$

as $x = a(1 - e^{-k_s t})$ where $k_s = \frac{S}{k_b}$

In both cases

$$\left(\frac{\partial x}{\partial a}\right)_t = \text{constant}$$

Thus this mode of entrance leads to the relationship actually found that $x \sim a$

This shows that when H_2S penetrates the protoplasmic surface in molecular form we shall get such linear curves as are found in Figs 3 and 4

The question may therefore be raised whether the entrance of H_2S follows the exponential law To decide this question the constant k has been calculated for Series I and II, according to the relationship

$$k = 2.3 \frac{1}{t} \log \frac{a}{a-x}$$

and the results have been plotted in Fig 5a Examination of the plot shows that k is not constant but that it falls off from a mean value of about 0.150 at the start to about 0.090 at the end of 10 minutes It appears therefore that the entrance of H_2S does not follow the simple "monomolecular" course However, if we plot the average "constants" up to 5 minutes against the equilibrium concentration

²² Since H_2S is a very weak electrolyte in aqueous solution it may be inferred that it will be still weaker in the non aqueous layer of the protoplasm, and that there will be a constant ratio of molecular H_2S in the aqueous phase to molecular H_2S in the non aqueous phase This is called S

we obtain Fig 5b This shows that the average "constant" is reasonably independent of the equilibrium concentration of H_2S in the sea water (which corresponds to a in the above equation) This, of course, is a characteristic of the monomolecular rate It appears therefore that we may be dealing with such a process, but with interfering

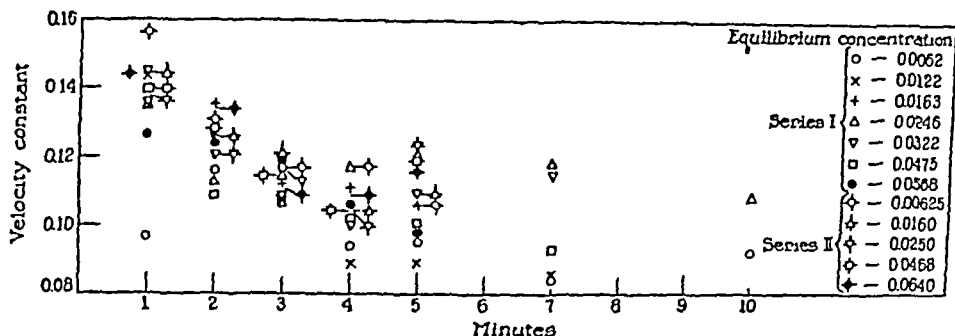


FIG 5a Change in the monomolecular velocity constant of H_2S entrance with time Constant calculated from the formula $k = 2.3 \frac{1}{t} \log \frac{a}{a-x}$, where a is the external concentration of molecular H_2S and x is the total sulfide ($\text{H}_2\text{S} + \text{S}^-$) in the sap

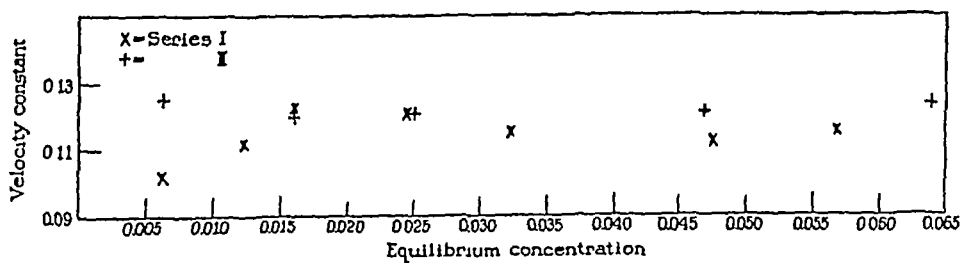


FIG 5b Plot of the "average" velocity constant from 1 to 5 minutes for each "equilibrium" concentration of H_2S , the latter being regarded as equivalent to the concentration of molecular H_2S in the external solution

factors which tend to make the calculation of the constant too high in the early part of the runs

Referring back to p 413, we find that the full expression for the relationship between x and a , taking both degree of dissociation and partition coefficients into account, is

$$x = \frac{S_0 a}{S(1 - \alpha)} (1 - e^{-k t})$$

or

$$k_1 t = \log \frac{S_0 a}{S a - S_1(1 - \alpha)x}$$

It will be clear that if $1 - \alpha$ is not unity x is not a true measure of the internal concentration of molecular H S. The pH however is decreasing with time and hence $1 - \alpha$ if it changes at all is increasing with time, \therefore the term $\frac{a}{a - x}$ calculated on the basis of $1 - \alpha = 1$ is too high at the start but with the discrepancy decreasing until $(1 - \alpha)$ does actually become unity. Such an adjustment of the value of this term would tend to decrease the early values of k but not the latter ones. However, it is inconceivable that $(1 - \alpha)$ will be constant for a constant value of t , no matter what the value of a ²³. But if it alters with a the relationship $\left(\frac{\partial x}{\partial a}\right)_t = \text{constant}$ cannot be true.

If however $S \approx S_1$ so that the simplified form

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

does not apply the value of the term $\frac{a}{a - x}$ will be changed.

To illustrate how this might affect the value of k let us consider a case where the external concentration of molecular H S is 0.010 normal and the ratio of $S - S_0$ is $1.0 \div 0.75$. For this case arbitrary values have been given to x from 0.001 to 0.005, and the corresponding values of t have been calculated from the formula

$$k = 2.3 \frac{1}{t} \log \frac{S_0 a}{S a - S_1 x}$$

For the first value of x , t is taken as 1 minute which fixes the value of k . Thus the calculations for the case have been idealized to make k constant.

This is compared with the value of k obtained for the case where S is taken as equal to S_1 and k has been calculated from the formula

$$k = 2.3 \frac{1}{t} \log \frac{a}{a - x}$$

the value of t being derived from the ideal case. The results have been collected in Table IV.

²³ Unless indeed it is unity for all values of a and t which seems to be approximately the case of H S in the concentration range studied by us.

This shows that failure to take into account the difference between S_o and S_i (the external partition coefficient S_o and the internal S_i) can yield a constant which is decreasing with time in a process which is nevertheless essentially monomolecular. And since S_o and S_i are not changing with time

$$\left(\frac{\partial x}{\partial a}\right)_t = \text{constant}$$

Moreover it will be clear that k is independent of a as is required by the monomolecular relationship

However, in these experiments it may be argued that the difference of the "partition coefficients" cannot be the cause of the fall of the

TABLE IV
Velocity Constants of H_2S Penetration for Ideal and Non-Ideal Cases
 $a = 0.010$

x	Ideal case					Non ideal case			
	$S_o a$	$S_o a - S_i x$	$\frac{\log \frac{S_o a}{S_o a - S_i x}}$	t	k	a	$a - x$	$\log \frac{x}{a - x}$	k
0.001	0.0075	0.0065	0.06215	1.000	0.143	0.010	0.009	0.04576	0.105
0.002	0.0075	0.0055	0.13470	2.167	0.143	0.010	0.008	0.09691	0.103
0.003	0.0075	0.0045	0.22185	3.569	0.143	0.010	0.007	0.15490	0.0998
0.004	0.0075	0.0035	0.33099	5.326	0.143	0.010	0.006	0.22185	0.0959
0.005	0.0075	0.0025	0.47712	7.677	0.143	0.010	0.005	0.30103	0.0902

constants because a was actually measured as x at equilibrium when according to the above formulation

$$S_i x = S_o a$$

and since in the adjusted constant of Table IV S_i was taken as unity the value actually measured was $S_o a$. Hence in the calculations from the actual experiments, Fig. 5a, k should have been constant.

It may be supposed also that $S_i < S_o$ so that if S_o is taken as unity $S_i a$ would be less than a . Following the same procedure in using this adjustment we find that its omission would cause the constant to increase with time, the opposite of the observed effect.

There may be other factors than the partition coefficients which should be taken into account.

Thus the constant k_4 in the simplest case where $S_o = S_i$, is a combination of several factors including the diffusion constants in the unstirred layers, the thickness of the layers, and the areas of the interfaces²⁴. It is scarcely likely that the slight difference in area between the internal and external layers in the same cell can have any effect, but it is entirely possible that the diffusion constants or the thicknesses or possibly both may be different. Just how these will affect the rate is not known. However, for the case of dried collodion Northrop²⁵ has shown that

$$\frac{dx}{dt} = \frac{DA}{h} (S_o a - S_i x)$$

where D is the diffusion constant in the collodion, A is the area of the interface, and h is the thickness. Applying this equation to each of the unstirred layers we get for the entrance of H_2S

$$\frac{dx}{dt} = \frac{D_o S_o A_o a}{h} - \frac{D_i S_i A_i (1 - \alpha) x}{h_i}$$

This can, of course, be integrated as before if we assume that all the terms are constant but t and x , to give the usual monomolecular formula. And although we do not know the values of any of the constants as was shown in Table IV an overall value can be given which will correct the drift in the value of k .

There remains, however, the possibility that the drift in k is due to differences in the permeability of the protoplasm and of the cellulose wall. This possibility is strengthened by the fact that in some experiments of the penetration of CO_2 into dead cells of approximately the same size and shape,²⁶ where the method of entrance is almost certainly by diffusion, the value of k also fell off with t .

²⁴ Osterhout W. J. V. *J. Gen. Physiol.*, 1932-33, 16, 529 footnote 31. Corresponding to the stirred non aqueous liquid between the unstirred layers we have in the case of *Valonia* a region between the sap and the external solution interfaces where there is probably some stirring due to protoplasmic movements. The rate of this stirring will as in the case of the model affect the rate of entrance, but we may assume that it will be about the same for all cells under the same conditions.

²⁵ Northrop J. H. *J. Gen. Physiol.* 1928-29, 12, 435. The terms used by Northrop have been translated by us to the terms used in this paper.

²⁶ Jacques A. G. and Osterhout W. J. V. *J. Gen. Physiol.*, 1929-30 13, 695

SUMMARY

The rate of entrance of H_2S into cells of *Valonia macrophysa* has been studied and it has been shown that at any given time up to 5 minutes the rate of entrance of total sulfide ($H_2S + S^{2-}$) into the sap is proportional to the concentration of molecular H_2S in the external solution

This is in marked contrast with the entrance of ammonia, where Osterhout has shown that the rate of entrance of total ammonia ($NH_3 + NH_4^+$) does not increase in a linear way with the increase in the external concentration of NH_3 , but falls off. The strong base guanidine also acts thus

It has been shown that the rate of entrance of H_2S is best explained by assuming that it enters by diffusion of molecular H_2S through the non-aqueous protoplasmic surface

It has been pointed out that the simple diffusion requires that the rate of entrance might be expected to be monomolecular. Possible causes of the failure of H_2S to follow this relationship have been discussed

A NOTE ON THE RELATION BETWEEN TOXICITY, RESISTANCE, AND TIME OF SURVIVAL*

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I

An interpretation of curves obtained in disinfection, hemolysis, and similar experiments was given in a previous paper. A general equation was offered which made the calculation of the time of survival possible if certain simple assumptions were made concerning the relation between toxicity and concentration.

One of the assumptions made was that the toxicity is proportional to the concentration. This assumption led to an equation which agreed with the experimental data in a narrow range only. There were also difficulties concerning the possible limits of integration in this case. The concentration can obviously be raised to any value before the time becomes 0. But in order that this may be possible according to the equation it had to be assumed that the resistance can possess negative values. The meaning of the resistance became thus rather vague if not absurd.

It is now realized that the general equation

$$t = -a \ln(h - r) + k \quad (1)$$

(t = time, h = toxicity, r = resistance, a and k are constants) given previously, is incompatible with the assumption that $h = f(c) \sim c$, because if r is constant t becomes negative when c approaches infinity. According to Equation 1, h must approach a finite value as c approaches infinity.

$$\lim_{h \rightarrow \infty} h = \lim_{f(c) \rightarrow \infty} f(c) = \text{const}$$

* Supplement to the paper which appeared in *J. Gen. Physiol.* 1934 17, 409

For $t = 0$ one obtains from Equation 1

$$h_{r=\text{const}} = a \ln(h_0 - r)$$

and

$$I_{h=\text{const}} = a \ln(h - r_0)$$

Since c approaches infinity when t approaches 0

$$h_0 = \lim_{c \rightarrow \infty} h_{c \rightarrow \infty}$$

It was found previously, that if $h \sim \frac{c}{c + \gamma}$ where $\gamma = \text{const}$, Equation 1 is in satisfactory agreement with the experimental results over the entire range tested so far. Now we find that this relation between toxicity and concentration is the simplest one compatible with Equation 1. Its meaning is that with increasing concentrations the toxicity approaches a limit because the organisms or certain elements indispensable for life in them, become saturated with the drug. On the basis of this relation between h and c

$$h_0 = \frac{c_{\infty}}{c_{\infty} + \gamma} = 1$$

and if r is constant and t and c vary

$$t_{r=\text{const}} = a \ln \frac{1 - r}{\frac{c}{c + \gamma} - r} \quad (2)$$

Now the rational assumption that $r_0 = 0$ becomes compatible with Equation 1 and we obtain for the case that c is constant and t and r vary

$$I_{c=\text{const}} = a \ln \frac{\frac{c}{c + \gamma}}{\frac{c}{c + \gamma} - r} \quad (3)$$

Formulas used in previous papers can be derived from these more general formulas and thus the numerical values obtained previously are correct

The function used to express the relation between binding (toxicity) and concentration goes over into a linear equation ($b \sim c$) only if $c < \gamma$ (Henry's law). This function, often considered to be characteristic of adsorption, holds for the distribution between two phases (volume as well as surface phases) if in one of the phases the volume of the bound substance is not negligible. The binding isotherm does not enable us to draw conclusions concerning the nature of binding. The applicability of Langmuir's equation indicates only that a saturation of the available space is approached if the concentration range used is wide enough. A discussion of this problem was given recently by Meyer and Hemmi.

It is well to emphasize that binding of a substance by a heterogeneous material of a complicated structure such as a living organism may take place to a different extent by different elements of the cell. Not all of this binding is necessarily pharmacologically effective (toxic). The toxic effect will depend on the importance of the physiological function of the cellular element which is affected by the binding. The element which is the most sensitive towards a drug has not necessarily the greatest affinity to it. Thus in certain cases it might be necessary to differentiate between the "effective part of the binding" and the "total binding". The effective fraction of the binding may vary with the concentration of the drug in the surrounding medium, for the isotherm of the binding, (e.g. adsorption isotherm) of a drug by different cellular elements will be as a rule different. The total binding is therefore not necessarily proportional to the toxicity.

II

We have attributed to each organism a property that it can bind a certain amount of a toxic agent and yet live indefinitely. The maximum amount still compatible with life is the resistance or threshold value. It is probable that this assumption is not strictly correct and that concentrations below that of the threshold value will also affect the organisms but will not kill them in times which would be comparable to those used in the experiments. The applicability of Equations 2 and 3 indicates that a "probable" threshold value (resistance) independent of the time can be attributed to each organism and that effects below this value are negligible as far as the observed phenome

non (death in a comparatively short time) is concerned. We have found a case since, (trivalent arsenicals on trypanosomes) in which a threshold value does not exist or else is a function of time.

The general objection was made (personal communication) that the theory given is too simple to account for the kinetics of a process as complicated as the death of an organism. It is believed that the fact that the resistance varies according to the law of probability actually proves that the biological processes resulting in resistance are of complex nature. It should be also noted that a manifestation of the complexity of the phenomenon is also the fact that the time of death is uncertain. It only has a probable value. The standard deviation from this value is great at low concentrations and decreases with increasing concentrations.

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CHEMICAL RESTORATION IN NITELLA

II RESTORATIVE ACTION OF BLOOD

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The irritability^{1 2} of *Nitella* depends on a substance (or a group of substances) which can be dissolved out of the protoplasmic surface by distilled water. From the water in contact with the cells substances can be extracted by organic solvents and these substances when re dissolved and applied to the cell restore the irritability. The experiments indicate that these substances are organic in nature.³

If this applies to irritability in general such substances must occur in animals. It is therefore of considerable interest to find that irritability and the potassium effect¹ can be quickly restored by blood.

Cells which had lost their irritability were placed in blood plasma. After 15 seconds many were found to be irritable when tested in the usual manner by applying an electrical stimulus (300 mv D.C.). In some cases a longer treatment⁴ (up to 2 minutes) was required.

In general the forms of the action current were like those found in control cells although in many cases they belonged to types which, though occurring in normal cells, are not those most commonly encountered in such cells.

Since it has been found¹ that calcium can restore irritability it was necessary to exclude its action. It was accordingly removed at the start by adding sodium oxalate (1 gm. to each liter of freshly drawn blood) after which the blood plasma was diluted with 4 parts of distilled water. As the inorganic substances remaining in the blood

¹ Osterhout, W J V. *J Gen Physiol* 1934-35 18, 987

² By this is meant the ability to give action currents as the result of electrical stimulation.

³ Osterhout, W J V, and Hill, S E. *Proc Soc Exp Biol and Med*, 1934-35, 32, 715, also S E. Hill unpublished results.

⁴ A much longer exposure resulted in some cases in a falling off of irritability.

after the removal of calcium have been found to be ineffective in restoring irritability and the potassium effect we may conclude that the active agents are organic substances ⁵

The experiments were performed on *Nitella flexilis* Ag at temperatures of from 20-22°C The technique employed was that described in a previous paper,¹ the action current and the responses to KCl were recorded photographically The cells were kept in distilled water until they no longer gave action currents when stimulated in the usual way with 100-400 mv d c This usually took 2 days After treatment with blood they were tested for irritability by the same electrical stimulus

The blood was drawn into vessels containing oxalate, placed in the centrifuge to remove blood corpuscles, and the plasma was diluted for use

There was some irregularity in the results, e g in some cases the first response was incomplete (i e the loss of p d amounted to from 10 to 50 per cent, instead of being complete as in the typical action current⁶) In such cases a second or third stimulus sometimes produced a complete response In a few cases three successive stimuli failed to elicit any response but it is possible that these cells were in poor condition

Human blood and that of the sheep, calf, and cat⁷ gave essentially the same results

What is said of irritability applies in general to the potassium effect Since the latter depends on the outer protoplasmic surface and the former more especially on the inner surface⁶ we might expect the potassium effect to be restored more promptly than irritability by the action of substances added to the external solution This seems to be true in general as has been stated¹ in describing experiments with NH₃, but in the case of blood there are many exceptions This is perhaps less surprising in view of the fact that the restorative substances in blood appear to penetrate with great rapidity ⁸ The fact that these sub-

⁵ This is confirmed by extraction experiments by S E Hill (unpublished results)

⁶ Osterhout, W J V, *J Gen Physiol*, 1934-35, 18, 215

⁷ In some cases whole blood diluted with 6 parts of distilled water to produce hemolysis (without oxalate) was employed with essentially the same results

⁸ When it is stated that irritability was restored after cells had been 15 seconds in blood plasma it must be remembered that after they had been removed and placed in contact with 0.01 M NaCl the restorative substances continued to diffuse inward (as well as outward) But in such cases the restoration was complete in less than a minute after the cells first came in contact with the blood plasma

stances are organic may help to explain their rapid penetration (it may be noted that chloroform is an example of an organic substance which penetrates with extreme rapidity⁹)

When an action current preceded the restoration of the potassium effect the latter was not regarded as due to the application of blood since the action current can itself cause the restoration of the potassium effect¹⁰

When a brief treatment with blood had failed to restore irritability replacement of 0.01 M NaCl by 0.01 M KCl gave the potassium effect in many cases thus showing that the restoration of the potassium effect by blood can be more rapid than the restoration of irritability

The substances which are responsible for the potassium effect and for irritability appear to be of widespread occurrence. The fact that they are found in animals suggests that they may be important in connection with the irritability of muscle and nerve and it seems possible that they may be concerned in various disturbances of nervous function. In that case it would be interesting to know how early they appear in animal ontogeny and phylogeny.

SUMMARY

Cells of *Nitella* exposed to distilled water lose their ability to produce action currents and to distinguish electrically between sodium and potassium. This ability was quickly restored by exposure to blood plasma deprived of calcium. Human blood and that of the cat, calf, and sheep gave essentially the same results.

The active agents appear to be organic substances.

⁹ Cf. Osterhout, W J V and Harris E S, *J Gen Physiol*, 1927-28, 11, 673

¹⁰ Osterhout W J V, and Hill S E, *J Gen Physiol*, 1934-35, 18, 681

SULFHYDRYL AND DISULFIDE GROUPS OF PROTEINS

II THE RELATION BETWEEN NUMBER OF SH AND S S GROUPS AND QUANTITY OF INSOLUBLE PROTEIN IN DENATURATION AND IN REVERSAL OF DENATURATION

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INTRODUCTION

In a previous paper methods were described for estimation of the sulfhydryl (SH) and disulfide (S S) groups of proteins. It was shown that in a denatured but unhydrolyzed protein the number of groups detectable is the same as that found in the hydrolyzed protein (Mirsky and Anson, 1934-35)¹. With the same methods, applicable to native as well as to denatured proteins, it is found that in some native proteins no SH and S S groups can be detected, while in others a fraction of the number present when the protein is denatured can be detected. In this paper it is shown that when a protein is partially denatured, that is when part of it is converted into a form insoluble under conditions under which the native protein is soluble, the insoluble fraction has the number of reactive SH and S S groups characteristic of completely denatured protein, whereas the soluble fraction has the number characteristic of protein which has not been denatured at all. When denaturation is reversed, when insoluble protein is converted into soluble protein, groups which have been detectable are so no longer. In the interfacial coagulation of a protein, that is when a film of insoluble protein forms at the surface of a protein solution, groups appear, the number detectable being the same as that found in the hydrolyzed protein. When a protein is coagulated by irradiation with ultraviolet

¹ This paper will be referred to as (Paper I)

light, groups are detectable in the insoluble protein, and the number of groups is not increased by subsequently exposing the insoluble protein to a typical denaturing agent, such as acid. Finally, when a protein is converted by urea into a form which has an increased number of S-S groups, that form is insoluble in a medium in which native protein is soluble. These new experimental results may be summarized by saying that, in denaturation, formation of insoluble protein and increase in detectable SH and S-S groups are closely linked phenomena. Denaturation, then, is a definite chemical reaction in which different quantitative methods give the same values for the extent of denaturation, a given protein molecule is either completely native or completely denatured. Another conclusion to be drawn from the experimental results now reported is that denaturation is a reversible reaction as indicated not only by changes in solubility but also by changes in the behavior of S-S groups. These conclusions are in harmony with our previous observations on hemoglobin and on trypsin. In the case of hemoglobin, the same results are obtained whether denaturation and its reversal are followed by changes in solubility, in spectrum, or in digestibility (Anson and Mirsky, 1933-34*b*). In the case of trypsin the same results are obtained whether denaturation and its reversal are followed by changes in solubility or in enzymatic activity (Anson and Mirsky, 1933-34*a*).

Appearance of Insoluble Protein and SH Groups in Denaturation by Heat

To investigate the appearance of groups in denaturation the denaturation of egg albumin by heat has been used. In this process both SH and S-S groups appear, but these observations have been limited to the former. Egg albumin solutions were heated so that varying portions (from 16 per cent to 83 per cent) were coagulated. The soluble and insoluble parts of these protein mixtures were separated and each fraction examined for SH groups. No SH groups could be detected in any of the fractions of soluble protein. The method used (Paper I) was to treat the protein with a cystine solution part of which is reduced to cysteine by protein SH groups if any are present. No cysteine was formed. In each of the insoluble fractions, on the other hand, SH groups were detectable. There were now,

indeed, no SH groups in the protein that were not detectable, for there were no additional groups that appeared on hydrolysis. This was demonstrated by treating the protein SH groups with cystine, hydrolyzing the protein, and then finding practically no cysteine in the hydrolysate. A control experiment showed the easily measurable amount of cysteine found in the hydrolysate of coagulated albumin if the treatment with cystine is omitted. Thus in the partial denaturation of egg albumin the soluble protein fractions contained no detectable SH groups, the insoluble fractions contained no SH groups that are not detectable.

Reappearance of Soluble Protein and Disappearance of S S Groups on Reversal of Denaturation

To study the behavior of S S groups during the reversal of denaturation serum albumin was used. It has been shown that it is possible to convert about 60 per cent of a denatured insoluble serum albumin preparation into a soluble form indistinguishable from native serum albumin (Anson and Mirsky, 1930-31). These experiments were repeated, and the number of S S groups estimated in native albumin, in denatured albumin, and in the mixture of reversed and non reversed albumin. In the denatured protein the number of groups is equal to the number found when the protein is hydrolyzed. Of these groups about 23 per cent are detectable in the native protein. On reversal some of the groups detectable in the denatured protein disappear, the number disappearing being equivalent to that which would be expected if the reversed albumin has the number of groups characteristic of native albumin. Since native serum albumin contains some detectable S S groups, it is apparent that presence of SH or S S groups does not in itself indicate that part of a protein system is in the denatured form. What can be said is that when the number of SH and S S groups increases or decreases that denaturation or reversal of denaturation has occurred.² Estimation of these groups can,

² Some proteins in contrast to egg albumin, while in their native state contain a certain number of detectable SH groups.

³ It will be shown in the following paper that this statement is true only if when the numbers of groups are estimated the various protein preparations are under the same conditions.

accordingly, serve as a valuable indication of the occurrence of denaturation in protein systems in which the usual test for denaturation, loss of solubility, is either inapplicable or inadequate

Appearance of SH Groups in Interfacial Coagulation

When a protein forms a unimolecular film at an aqueous interface, it loses its solubility in water, for if the film is dispersed the protein does not redissolve. If before the film forms, the protein is readily soluble in water, some change in the protein must have occurred during formation of the film. In this respect the "spreading" of a protein film differs from that of an insoluble fatty acid, for when the latter spreads there is no reason to suppose that a change in its constitution occurs. Investigators of protein films, interested mainly in the size and shape of the protein molecule in a film, have paid little attention to possible changes in constitution of the protein. Since the protein in a film is insoluble, coagulated, it is important to compare its chemical properties with those of proteins rendered insoluble by denaturing agents such as heat and acid. For this purpose egg albumin is a suitable protein since it readily coagulates at an air-water interface. A large quantity of coagulated egg albumin is obtained by shaking an albumin solution. As soon as a film forms, it is dislodged by the agitation, leaving place for a new film to form. To estimate the number of detectable SH groups in the insoluble protein, the method (Paper I) used was to treat it with iodoacetate which reacts with SH groups if any are present. The excess of iodoacetate is removed, the protein is hydrolyzed, and the cysteine content of the hydrolysate is estimated. The cysteine content of protein that has not been treated with iodoacetate is also estimated. The decrease in cysteine content of protein treated with iodoacetate is a measure of the number of its SH groups that reacted with iodoacetate. In native egg albumin no SH groups react with iodoacetate, in insoluble albumin all the SH groups contained in the molecule react with iodoacetate, for in the hydrolysate practically no cysteine is found. When ferricyanide is used to react with SH groups, the same results are obtained. With respect to activity of its SH groups, then, egg albumin coagulated at an interface is the same as egg albumin coagulated by typical denaturing agents.

Appearance of Insoluble Protein and SH Groups in Denaturation by Ultraviolet Light

In egg albumin denatured by ultraviolet light, the nitroprusside test shows that SH groups are present. Our experiments demonstrate that in egg albumin denatured in this way, all the SH groups contained in the molecule are detectable, for no increase in the number of detectable groups occurs on adding an excess of acid. The protein rendered insoluble by irradiation with ultraviolet light is treated with iodoacetate, as the result of which the nitroprusside test fails to show any SH groups, and none become detectable after adding an excess of trichloroacetic acid.

Appearance of S-S Groups and of Insoluble Protein in Denaturation by Urea

Denatured protein formed in a number of different ways—by heat, acid, alkali, alcohol, surface action, ultraviolet light—manifests SH and S-S groups. After it had been found that urea denatures proteins (Ramsden, 1902, 1913, Anson and Mirsky, 1929–30), Hopkins (1930) observed that in these denatured proteins too, the groups are present. Since so many different agents cause a loss of solubility which is accompanied by appearance of SH and S-S groups, the loss of solubility and the appearance of groups appear to be closely linked. In the denaturation of serum albumin by urea, however, Hopkins believes that he has caused SH groups to appear without the formation of insoluble protein. We have found that the formation of insoluble protein can be demonstrated if the test is properly carried out.

Hopkins observed that when a concentrated urea solution is added to serum albumin there is a prompt appearance of S-S groups but that on removal of the urea by dialysis no insoluble protein can be detected. From this observation, which we have confirmed, he concluded that appearance of S-S groups and appearance of insoluble protein are not necessarily correlated. It should be noted that Hopkins tested for S-S groups *before* the removal of urea and for insoluble protein *after* the removal of urea. We have observed that if the solution of serum albumin in urea is poured into a large volume of concentrated salt solution (in which native serum albumin is soluble) 95 per cent of the protein precipitates. That is, if the tests for S-S groups and insoluble

protein are both made under the same conditions before the removal of urea, then no evidence can be found for the appearance of S-S groups without the appearance of insoluble protein. The reason Hopkins could detect no insoluble protein *after* dialysis (in our experiments a small part of the protein precipitated during dialysis) is that when urea is removed much of the denatured insoluble protein changes into native soluble albumin just as hemoglobin denatured by salicylate (which acts like urea) changes into native soluble hemoglobin when the salicylate is removed by dialysis (Anson and Mirsky, 1933-34*b*)

EXPERIMENTAL

I The Heat Denaturation of Egg Albumin

(a) *Partial Coagulation*—A thick paste of egg albumin crystals in ammonium sulfate was diluted with water to obtain a solution containing 0.05 gm. of albumin per cc. For each experiment 30 cc. of this solution were transferred to a 250 cc. centrifuge flask and diluted with 20 cc. water and 20 cc. of a solution prepared by dissolving 20 gm. Na_2SO_4 in 100 cc. of $\text{M}/5$ pH 4.8 acetate buffer. The sodium sulfate-acetate mixture was used to ensure complete precipitation of denatured albumin. The flask containing the albumin solution was placed in a water bath kept at 95°C . As coagulation proceeded the albumin mixture was gently stirred. Times of heating were adjusted so that the quantities of coagulated protein varied from 16 to 83 per cent of the total protein present. After heating, the albumin mixture was cooled under the tap and centrifuged. The supernatant fluid was filtered to remove the few floating particles usually present.

(b) *SH Groups of Uncoagulated Protein*—To the clear filtrate was added an equal volume of saturated ammonium sulfate to precipitate completely the native albumin remaining in solution, and the suspension was filtered. The precipitated albumin, scraped off the filter paper, and transferred to a 50 cc. centrifuge tube, was now ready for estimation of SH groups. No more than approximately 400 mg. of this protein were used. Estimation of SH groups was by the "direct" method (Paper I) in which the protein is mixed with an excess of a cystine solution part of which is reduced to cysteine if reactive protein SH groups are present. The quantity of cysteine formed is equivalent to the number of protein SH groups present. This method, previously used for denatured proteins, may be used for native proteins if the precaution is taken of not making the cystine solution employed so alkaline as to denature the protein. Sodium hydroxide was accordingly added to the cystine until the solution was definitely blue to thymol blue but colorless to thymolphthalein.

(c) *SH Groups of Coagulated Protein*—The coagulated albumin remaining in the flask after centrifuging was thoroughly washed to free it of any adhering native

protein To do this the precipitate was mixed with 200 cc. of water and 15 cc of the sodium sulfate acetate mixture stirred mechanically for 30 minutes and then centrifuged When the supernatant fluid no longer gave a precipitate on addition of 20 cc of a 50 per cent trichloroacetic acid solution washing was stopped To test for SH groups a portion of the precipitate containing at the most 700 mg of albumin was transferred to a 250 cc centrifuge flask, stirred with 200 cc of water, and 40 cc of saturated $(\text{NH}_4)_2\text{SO}_4$, centrifuged and the supernatant fluid discarded The albumin was suspended in 200 cc of a cystine solution like that used for estimating SH groups and was gently stirred for an hour when 25 cc of 50 per cent CCl_3COOH were added The mixture was centrifuged the supernatant fluid being discarded The precipitated albumin was washed three times with water and trichloroacetic acid dried hydrolyzed, and finally the cysteine content of the hydrolysate was estimated The total quantity of albumin that had been coagulated consisted of the part which was treated with cystine and then dried and of the part which was not treated with cystine The former was weighed and the latter was estimated colorimetrically with the phenol reagent

In addition to testing the SH groups of the part of the albumin which had been coagulated by heat and the part of the albumin which was still soluble we estimated by the direct cystine reduction method the SH groups of albumin made completely insoluble by heat and we estimated by the colorimetric method previously described (Paper I) the total cysteine content of the hydrolysate of albumin coagulated by trichloroacetic acid

RESULTS

The cysteine content of hydrolyzed egg albumin was 0.616 per cent Heat coagulated egg albumin contained 0.56 per cent of SH groups SH groups are recorded in terms of cysteine, that is as the quantity of cysteine which would have the same sulfur content, the amount being expressed as per cent of the total amount of protein In experiments on partial heat denaturation the quantity of insoluble protein formed varied from 16 to 83 per cent In no case could any SH groups be detected in the soluble albumin fraction, that is, none of the cystine with which the soluble albumin was treated was converted into cysteine The insoluble fractions, treated with cystine and then precipitated, washed, and hydrolyzed, formed with phosphotungstate blue colors so faint that the cysteine contents of the hydrolysates could not be estimated accurately, an indication that practically all of the SH groups contained in the protein had been oxidized by the cystine and hence were present in the insoluble, but unhydrolyzed, protein

II Reversal of Denaturation of Serum Albumin

Native, denatured, and reversed horse serum albumin were prepared as described by Anson and Mirsky (1930-31). A precipitate of denatured albumin was prepared in the following manner: 400 mg of the acid-acetone powder of albumin were dissolved in 20 cc of water. The solution was immersed in boiling water until its temperature reached 95°C when to it were added a mixture at 100°C containing 40 cc M/5 acetate buffer of pH 4.8 and 2.6 cc N/5 NaOH. The albumin suspension was cooled rapidly to about 40°C under the tap, divided into 2 equal parts, and centrifuged. The supernatant fluids, in which only a slight haze appeared on addition of trichloroacetic acid, were discarded.

The denaturation of one sample of precipitated albumin was partially reversed as follows. The precipitate was suspended in 10 cc of water, and a clear solution was obtained by adding 3 cc N/5 hydrochloric acid. To the solution just enough N/5 NaOH was slowly added to re-dissolve the precipitate of albumin that gradually formed. The solution was now blue to brom-thymol-blue and faintly red to phenol red.

The number of S-S groups of partially reversed and of non-reversed albumin was now estimated. For this purpose the first step was to reduce the protein with thioglycolic acid. After reduction SH groups were detectable in the protein whereas originally none was present, and the number of SH groups formed was a measure of the number of S-S groups present in the protein. SH groups formed were estimated by hydrolyzing the protein and estimating the cysteine content of the hydrolysate. The hydrolysate of serum albumin not treated with thioglycolic acid contains no cysteine. The quantity of cysteine in the hydrolysate treated with thioglycolic acid was therefore equivalent to that portion of the cystine of the protein which was originally present as reducible S-S groups in the unhydrolyzed protein. The partially reversed albumin was reduced under precisely the same conditions. To the precipitate of denatured protein were added 15 cc of a concentrated sodium sulfate solution kept at 30°C (this solution, to be referred to again, contained 40 gm of the anhydrous salt dissolved in 100 cc of water), and in the solution of reversed protein, warmed to 30°C were dissolved 6 gm of anhydrous sodium sulfate. A neutralized thioglycolic mixture was made at 30° by adding to 2 cc of acid enough 0.4 N KOH (dissolved in concentrated sodium sulfate) to make the solution just red to phenol red and then an additional 5 cc of alkali, 15 cc M/1 pH 7.3 $\text{K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer, 45 cc concentrated sodium sulfate, and finally 6 gm of anhydrous sodium sulfate. One-half of this mixture was added to the partially reversed serum albumin and the other half to the non-reversed albumin. The protein suspensions, contained in 100 cc stoppered Erlenmeyer flasks, were kept at 30° with frequent agitation for 30 minutes. At the end of this time the proteins were freed of thioglycolic acid by prolonged washing with trichloroacetic acid (Paper I). Trichloroacetic acid was removed from the precipitates by transferring them to 30 cc collodion tubes and dialyzing in a rocking dialyzer for 5 hours against 0.005 N HCl. It was

necessary to remove trichloroacetic acid because otherwise on subsequent addition of acetone, part of the albumin would dissolve. The proteins were then dehydrated in acid acetone, dried, hydrolyzed and the cysteine contents of the hydrolysates were estimated by the method previously described (Paper I).

The S-S groups of native serum albumin were estimated in the same way as were those of denatured and partially reversed protein. During the reduction process native albumin was precipitated by sodium sulfate at 30°.

The quantity of albumin that becomes soluble on reversal was estimated in the manner previously described (Anson and Mirsky, 1930-31).

RESULTS

S-S groups are recorded in terms of cystine, that is as the quantity of cystine that would have the same sulfur content, the amount of cystine being expressed as per cent of the total amount of protein.

1 Cystine content of serum albumin—5.6 per cent

2 S-S groups of denatured, non reversed serum albumin—5.46, 5.21, 6.08, 6.03—average 5.7 per cent

3 S-S groups of partially reversed serum albumin—3.37, 2.81, 2.97—average 3.05 per cent

4 S-S groups of native serum albumin—1.42 per cent

5 S-S groups appearing on denaturation, (2) minus (4)—4.28 per cent

6 S-S groups disappearing on partial reversal, (2) minus (3)—2.65 per cent

7 Per cent of the S-S groups appearing on denaturation that disappear on partial reversal, 6.5—6.2 per cent

8 Per cent of the denatured insoluble protein that becomes soluble on reversal—65 per cent

III Surface Coagulation of Egg Albumin

Coagulated albumin was prepared by dissolving an ammonium sulfate precipitate of crystalline egg albumin, which contained about 5 gm of protein in 500 cc of water and shaking the solution in a 2 liter bottle. The bottle was shaken in a cold room at about 5° for 20 hours. Coagulation was complete for when a little of the suspension was filtered it was found that no precipitate appeared in the filtrate on addition of trichloroacetic acid. The suspension was centrifuged, most of the protein forming a cake at the surface. By rubbing the precipitate with a rubber policeman, its physical state was changed so that when suspended in water and centrifuged nearly all of it settled to the bottom of the flask.

The precipitate was divided into three approximately equal parts, one of which

was washed free of salt with trichloroacetic acid, dehydrated, and dried. Each of the other parts of the precipitate was placed in a 250 cc centrifuge bottle and suspended in 100 cc $M/5$ pH 9.6 borate buffer. To one suspension was added 15 cc $M/2$ potassium ferricyanide and to the other 25 cc of $M/2$ sodium iodoacetate (iodoacetic acid neutralized to phenol red with NaOH). After standing for $1\frac{1}{2}$ hours with occasional agitation both bottles were filled with water and centrifuged. The supernatant fluids, in which no precipitates appeared on addition of trichloroacetic acid, were discarded. The albumin that had been treated with iodoacetate was washed free of iodoacetate with trichloroacetic acid, dehydrated, and dried. The albumin containing potassium ferricyanide was washed until the yellow color of ferricyanide disappeared. This was done by stirring the precipitate with 250 cc of water, adding 1 cc of saturated sodium chloride solution to accelerate sedimentation of the precipitate, centrifuging, and then stirring with water again. After removal of ferricyanide the albumin was washed with trichloroacetic acid to remove salt, dehydrated, and dried.

Native egg albumin was treated with iodoacetate and with ferricyanide. In 200 cc of an $M/5$ pH 9.6 borate buffer 2 gm of albumin were dissolved. To half of this solution was added iodoacetate and to the other half was added ferricyanide in the concentrations described above. After standing $1\frac{1}{2}$ hours the extent to which the protein SH groups had reacted was determined. In the preparation containing ferricyanide this was quickly done by testing for ferrocyanide. None was present for when ferric chloride was added no prussian blue appeared.⁴ The albumin treated with iodoacetate was diluted to 200 cc with water and then precipitated by addition of trichloroacetic acid. The precipitate was washed with trichloroacetic acid, dehydrated, and dried.

RESULTS

The various dried albumin preparations which have been described were hydrolyzed and the cysteine contents of the hydrolysates were estimated. The diminution in cysteine content of those preparations treated with iodoacetate and ferricyanide is a measure of the number of reactive SH groups, which are recorded in terms of cysteine.

- 1 Cysteine content of egg albumin—0.59 per cent
- 2 Cysteine content of egg albumin coagulated by shaking—0.55 per cent
- 3 Cysteine content of coagulated egg albumin, after reacting with iodoacetate—too little for accurate estimation
- 4 Cysteine content of coagulated egg albumin after reacting with potassium ferricyanide—too little for accurate estimation

⁴ A detailed description of this test will be described in a forthcoming paper.

5 Cysteine content of native egg albumin after reacting with iodoacetate—0.57 per cent

6 SH groups of coagulated egg albumin, that is, (2) minus (3) or (4)—0.55 per cent

7 SH groups of native egg albumin as shown by the reaction with iodoacetate, that is, (1) minus (5)—none

8 SH groups of native egg albumin, as shown by the reaction with ferricyanide—none, for no ferrocyanide was formed

IV Coagulation of Egg Albumin by Irradiation with Ultraviolet Light

A 1 per cent albumin solution was prepared by dissolving an ammonium sulfate precipitate of crystalline egg albumin in water. 10 cc. of the solution in a shallow layer were radiated with a mercury vapor lamp until approximately one half of the albumin was coagulated. During the radiation the temperature of the protein solution was not allowed to exceed 35°. The coagulated protein was freed of soluble protein in the manner described above, in Section I c. When tested with nitroprusside and ammonium hydroxide a portion of the coagulated protein gave an intense reaction for SH groups. The rest of the coagulum was treated with iodoacetate the excess of which was removed by repeated washing with trichloroacetic acid. The precipitate was neutralized by suspending it in 50 cc. 0.1 M/10 pH 7.0 phosphate buffer. After centrifuging, no SH groups could be detected in the protein when tested with nitroprusside and ammonium hydroxide.

V Denaturation of Serum Albumin by Urea

A preparation of horse serum albumin (Anson and Mirsky, 1930-31) dialyzed free of ammonium sulfate contained 6.5 per cent albumin. In 1 cc. of this solution 1 gm. of urea was dissolved and the solution was allowed to stand for 4½ hours at room temperature. 20 gm. of anhydrous sodium sulfate were dissolved in 100 cc. of a pH 4.8 0.1 M/10 acetate buffer. The urea albumin solution was added to 40 cc. of the sodium sulfate solution. The precipitate obtained was removed by filtration. To 10 cc. of the filtrate were added 3 cc. of 20 per cent trichloroacetic acid and the suspension was centrifuged. The supernatant fluid which gave no precipitate on further addition of trichloroacetic acid was discarded. The quantity of albumin in the precipitate was estimated by the phenol reagent colorimetric method using albumin as a standard. It was 5.5 per cent of the quantity mixed with urea. Urea had rendered insoluble 94.5 per cent of the albumin. When 1 cc. of the original native albumin solution was added directly to 40 cc. of the sodium sulfate solution no precipitate was obtained.

CONCLUSIONS

1 In native egg albumin no SH groups are detectable, whereas in completely coagulated albumin as many groups are detectable as are

found in the hydrolyzed protein. In egg albumin partially coagulated by heat the soluble fraction contains no detectable groups, and the insoluble fraction contains the number found after hydrolysis.

2. In the reversal of denaturation of serum albumin, when insoluble protein regains its solubility, S-S groups which have been detectable in the denatured protein, disappear.

3. When egg albumin coagulates at an air-water interface, all the SH groups in the molecule become detectable.

4. In egg albumin coagulated by irradiation with ultraviolet light, the same number of SH groups are detectable as in albumin coagulated by a typical denaturing agent.

5. When serum albumin is denatured by urea, there is no evidence that S-S groups appear before the protein loses its solubility.

6. Protein denaturation is a definite chemical reaction. Different quantitative methods agree in estimates of the extent of denaturation, and the same changes are observed in the protein when it is denatured by different agents. A protein molecule is either native or denatured. The denaturation of some proteins can be reversed.

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SULFHYDRYL AND DISULFIDE GROUPS OF PROTEINS

III SULFHYDRYL GROUPS OF NATIVE PROTEINS—HEMOGLOBIN AND THE PROTEINS OF THE CRYSTALLINE LENS

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In a denatured but unhydrolyzed protein the number of SH and S S groups detectable is equivalent to the quantity of cysteine and cystine found in the hydrolyzed protein (Mirsky and Anson, 1934-35)¹ Detectable protein SH groups react with iodoacetate or with oxidizing agents such as ferricyanide and cystine. Detectable protein S S groups have the property of being reduced to SH groups by a thiol compound such as thioglycolic acid. The methods of detection of these groups are applicable without any modifications to native as well as denatured proteins. When these methods are applied to native proteins which are under the same conditions as are the denatured proteins, some (egg albumin, for instance) manifest no groups at all, while others (serum albumin) show only a fraction of the number present in the denatured form.

Since the typical protein, egg albumin, has no detectable SH groups, it might be suspected that the S S groups detected in supposedly native serum albumin (Mirsky and Anson, 1935-36) are present only because the procedure for detecting groups has caused some denaturation. In the present investigation, however, SH groups are demonstrated in native hemoglobin, and in this case there is some assurance that the procedure does not cause denaturation, for denatured hemoglobin can readily be detected spectroscopically (Anson and Mirsky, 1925, 1928-29). The disadvantage of using hemoglobin, that heme interferes with estimation of SH groups, can be avoided.

¹ This paper will be referred to as (Paper I)

A study of the SH groups of native hemoglobin shows that the number of groups detectable is dependent on hydrogen ion concentration, the number increasing as the pH rises. At pH 6.8 no groups are detectable but as the pH is raised (in our experiments as far as 9.5) more and more groups appear. When the pH is brought back to 6.8 they are no longer detectable.

These observations raise the question as to how the SH groups of native hemoglobin differ from those of denatured hemoglobin. The difference between the groups of native and denatured proteins is that the latter can be detected at a pH at which the former cannot be. It is found that at pH 6.8, where no groups are detectable in native hemoglobin, SH groups are detectable in denatured globin, and the number detectable is the maximum available, that is, it is equal to the number of cysteine molecules found in hydrolyzed globin. In comparing native hemoglobin with denatured globin, the differences observed may in part be due to the presence or absence of heme as well as to the state of the protein. The same differences, however, are observed between the native and denatured forms of other proteins, the proteins of the crystalline lens, for instance. In general, then, the effect of denaturation is to extend towards the acid side the pH range in which SH groups are detectable. In every thiol compound (thioglycolic acid, cysteine, and glutathione, for example) activity of the SH group in its reactions with oxidizing agents or with iodoacetate increases with a rise in pH, and in this respect both native and denatured proteins resemble other thiol compounds. But even without change in pH, the SH groups of a protein can be activated by denaturation.

The experiments on hemoglobin and the lens proteins show that activation of SH groups can serve as a satisfactory criterion of denaturation. The test for SH groups activated by denaturation should be carried out at a pH so low that the groups of native protein are inactive and yet high enough for those of denatured protein to be active. Proper conditions for the test vary accordingly from one protein to another, for each protein has its characteristic curve relating pH to activity of groups. The SH groups of the native lens proteins become active at a pH below 6.8, those of hemoglobin above pH 7.0, and those of egg albumin are inactive even at pH 9.6.

SH Groups of Hemoglobin

The method used for estimating the SH groups of hemoglobin is substantially the same as the "indirect" method of Mirsky and Anson (Paper I). Hemoglobin at the desired pH is treated with potassium ferricyanide² to oxidize any SH groups present, and the excess ferricyanide is removed by dialysis. Globin and heme are then separated by the acid acetone procedure (Anson and Mirsky, 1929-30) so that the heme will not interfere with the subsequent analytical procedure for oxidized heme reacts with thiol compounds. The SH groups of the denatured globin prepared in this manner are estimated and compared with the number found in globin prepared from hemoglobin which was not treated with ferricyanide. The difference between these values is equal to the number of SH groups oxidized by ferricyanide, and it is accordingly a measure of the number of SH groups present in hemoglobin under the conditions of the experiment.

The number of SH groups found in native hemoglobin is dependent upon the hydrogen ion concentration. At pH 6.8 almost no groups are detectable, in a pH 7.3 buffer, 28 per cent of the total number of groups contained in the protein appear, in pH 9.0, 44 per cent, and in pH 9.5, 65 per cent are found. The effect of change in pH is reversible. If the pH is brought to 8.75 and then, after an interval to 6.8, no groups are detectable at the latter pH.

The experiments at different hydrogen ion concentrations show that the iron porphyrin part of the hemoglobin molecule can be oxidized independently of its SH groups, for at pH 6.8 ferricyanide oxidizes all of the hemoglobin to methemoglobin without oxidizing any of the SH groups. And, conversely, it is possible to oxidize SH groups of hemoglobin without oxidizing other parts of the molecule. This can be done at pH 9.6 by using cystine, a very mild oxidant, to oxidize SH groups without simultaneous formation of methemoglobin.

When the SH groups of hemoglobin are oxidized at pH 9.5, either by ferricyanide or cystine, no denatured protein is present by spectroscopic test (Anson and Mirsky, 1925, 1928-29). If ferricyanide is

² The potential of the ferrocyanide/ferricyanide system is constant in the pH range used in these experiments.

used, methemoglobin is formed, and if cystine is used, the hemoglobin remains unchanged spectroscopically, in neither case is any parahematin or hemochromogen, that is denatured hemoglobin, observed spectroscopically

SH Groups of the Proteins of the Crystalline Lens

The lens proteins are treated with iodoacetate which destroys active SH groups and the excess iodoacetate is removed simply by precipitating the protein with trichloroacetic acid and washing. In this

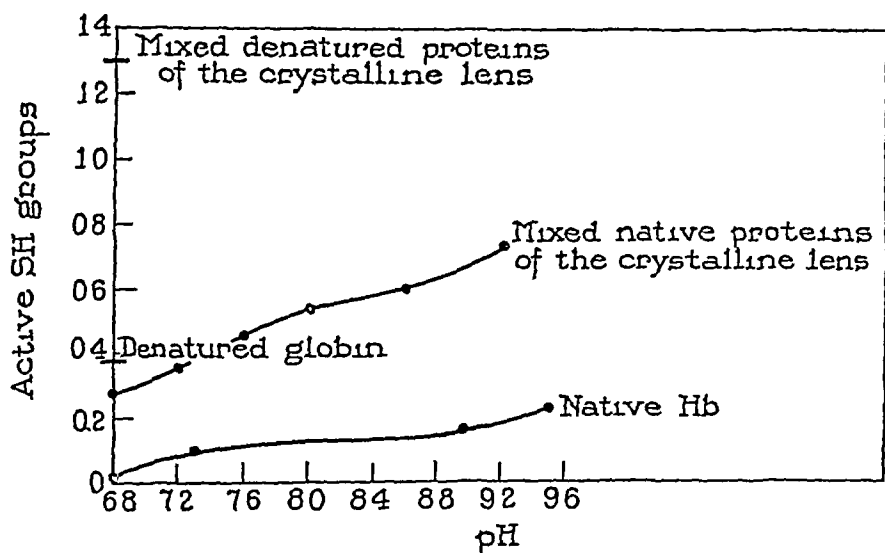


FIG 1 Relation between pH and number of active SH groups in hemoglobin and in the mixed proteins of the crystalline lens

process the protein is denatured, but in presence of acid the SH groups of denatured proteins are inactive. The number of groups that react with iodoacetate is found by hydrolyzing the protein and estimating its cysteine content. The difference between the cysteine contents of proteins treated with iodoacetate and of those not treated is a measure of the number of SH groups that react with iodoacetate. When these proteins are said to be in the native state, it is meant that in their preparation no agent known to denature proteins is employed. Evidence, such as that available for hemoglobin preparations, that no denatured protein is present, is lacking. And yet a study of the

activity of SH groups indicates that there is little if any, denatured protein in preparations of the lens proteins, for behavior of the groups is similar to that of the SH groups of native hemoglobin. Activity of the SH groups of the native lens proteins is augmented as the pH rises. The effect is reversible, groups activated by a rise in pH lose their activity when the pH drops. When these proteins are denatured, their SH groups become fully active at a pH at which groups of the native proteins are only just beginning to be active.

EXPERIMENTAL

The SH groups of globin were first detected by means of the nitroprusside reaction (Anson and Mirsky, 1930-31). It was then recognized by Schüler (1932) that in the reaction between hemoglobin and ferricyanide these groups, as well as heme, might react with ferricyanide. To estimate the number of SH groups of hemoglobin that might take part in this reaction Schüler titrated globin with ferricyanide. It was assumed that ferricyanide would react only with the SH groups of globin. His experiments have been repeated and his observations confirmed; furthermore, his estimate of the number of SH groups in globin (of the guinea pig) is the same as those we have made (of horse globin) by entirely different methods which appear to be specific for SH groups. When, however, other proteins are treated with ferricyanide we find that there are reducing groups in addition to the SH which react with ferricyanide. These hitherto unrecognized reducing groups of proteins will be described in another paper. Another assumption made by Schüler was that in globin and hemoglobin the same number of SH groups react with ferricyanide. It is shown in this paper that only part of the SH groups of globin are active in hemoglobin. The number active increases as the pH rises. The behavior of the SH groups of hemoglobin should not be neglected in studies on the oxidation-reduction potential of the hemoglobin-methemoglobin system.

It has been claimed by Meldrum (1932) that neither SH nor S-S groups are detectable in globin and that the observations of Anson and Mirsky can be explained by a failure to distinguish between the color reaction given by nitroprusside with acetone, and that given with SH groups. The evidence for the existence of SH and S-S groups in globin may be briefly summarized.

1. Horse globin prepared by the acid-acetone procedure and then thoroughly washed with trichloroacetic acid to remove acetone gives a marked color reaction with nitroprusside and ammonium hydroxide. This color is distinctly different from the color given by acetone. Serum albumin prepared by the acid-acetone procedure and then washed free of acetone does not give a color reaction with nitroprusside and ammonium hydroxide; neither does it contain SH groups when tested by other methods. This color reaction of horse globin is unmistakable. Only if insufficient care is taken in preparing globin in which case a deeply pig-

mented protein instead of a colorless one is obtained, are these reactions obscure, as stated by Meldrum

Globin was treated in the following manner before being tested with nitroprusside, about 100 mg of protein, with acetone still adhering to it, were mixed with a little water, so as to form first a thick and then a thin paste. About 200 cc of water were then added and the mixture stirred mechanically for 15 minutes, when 20 cc of a 50 per cent solution of trichloroacetic acid were added. After centrifuging the supernatant fluid was discarded, and the protein was washed again in the same manner. This process was repeated four times. The protein was then washed with sodium sulfate and a pH 7.3 $\text{KH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$ buffer as described below (p 447). After centrifuging, a little of the protein (now in the form of a very thick paste) was placed on a piece of folded filter paper. The protein was moistened with a few drops of a 5 per cent solution of sodium nitroprusside, the filter paper absorbing the excess fluid. The protein was now moistened with a few drops of dilute ammonium hydroxide.

2 Denatured horse globin reduces cystine to cysteine. This reaction, which is certainly not due to acetone, depends on the presence of SH groups in the protein. The experimental procedure for this reaction is described below.

3 After horse globin has been oxidized with ferricyanide, as described below, it gives no test with nitroprusside for SH groups but it now gives an intense test for S-S groups. Globin treated with ferricyanide does not reduce cystine. To test for S-S groups a few small crystals of potassium cyanide were placed on the protein (on a piece of filter paper, as in the test for SH groups) before adding nitroprusside. No ammonium hydroxide was used (Walker, 1925).

4 When hydrolyzed horse globin reacts with phosphotungstate a blue color is formed indicating the presence of cysteine. Globin oxidized by ferricyanide and then hydrolyzed contains no cysteine. This indicates that before oxidation globin contained a number of SH groups equivalent to the cysteine content of non-oxidized hydrolyzed globin (Paper I).

5 If horse globin is dried in an oven at 110° , in the course of many days its SH groups, as tested for by nitroprusside, gradually disappear, the test for S-S groups does not become negative.

6 Ox globin was prepared by Wu's method, which does not involve the use of acetone, but in which there is ample opportunity for oxidation of SH groups. This globin, when treated with nitroprusside, gives no test for SH groups but does give an intense test for S-S groups.

7 If ox globin, prepared by Wu's method, is treated with acetone which is then washed away, the protein does not now appear to contain SH groups due to traces of acetone which, it may be imagined, were not removed. It gives no color reaction with nitroprusside and ammonium hydroxide, although the protein, due to the treatment with acetone, is so white that even a faint color could be detected. This protein gives an intense test for S-S groups.

Haurowitz (1935) believes that globin contains SH groups, although he was unable to obtain a test with nitroprusside using either ammonium hydroxide or

potassium cyanide. He has suggested that it may be through its thiol groups that globin is attached to heme. This theory, for which Haurowitz advanced no convincing evidence whatsoever, is untenable for several reasons.

1 More than half of the SH groups of globin can be oxidized while the globin is joined to heme in the form of hemoglobin without disrupting the molecule.

2 A molecule of globin of the horse contains only two SH groups although it can combine with four heme molecules.

Vickery and White (1933) have devised a method for estimating the cystine content of proteins that is entirely different from the method used in our investigations. In their method no distinction is made between cystine and cysteine; quantities of both are lumped together as 'cystine content'. Their estimate of the 'cystine' content of horse hemoglobin (0.41 per cent) is in good agreement with our value for the *cysteine* content of horse globin (0.42 per cent). This agreement indicates that horse globin contains cysteine but not cystine. Our estimate of cystine content, however, does not agree with that reported by Vickery and White. Since horse globin contains cysteine it is to be expected (Mirsky and Anson, 1930) that an estimation of its cystine content by the Folin Marenz (1929) method would be too high, for Folin and Marenz assume that cysteine and cystine have the same color value with their reagent, whereas cysteine actually gives twice as much color as cystine. (The estimate is 1.14 per cent.) But even after all of the cysteine of globin has been oxidized to cystine, its cystine content as given by a modification (Paper I) of the Folin Marenz method is 0.63 per cent, distinctly higher than the value reported by Vickery and White.

Cystine may not be the only substance in a hydrolysate of oxidized globin that reacts with the phosphotungstate of Folin and Marenz in presence of sulfite. That in the hydrolysates of a number of proteins no other substance is present, is shown by the evidence presented in a previous paper (Paper I) and also by the agreement in estimates of cystine content of several proteins by the Folin Marenz and the Vickery White methods. And yet there is evidence that under certain conditions protein hydrolysates do contain interfering substances. We find using the Folin Marenz method, that the cystine content of serum globulin is 2.2 per cent, which is in fair agreement with the value (1.82 per cent) obtained by the Sullivan method.³ In hydrolyzing the protein 6*N* H₂SO₄ was used. If more concentrated acid is used the estimate of cystine content using the Folin Marenz reagent rises to 3.0 per cent if 10*N* H₂SO₄ is used and to 3.4 per cent if 11.3*N* H₂SO₄ is used.⁴ These results suggest that when globin is hydrolyzed

³ Our thanks are due to Dr. Sullivan for the analysis by his method (1926).

⁴ These experiments were carried out because of the estimates of cystine content of serum globulin reported by Tuchman and Reiner and by Reiner and Sobotka using a modification of the Folin Marenz method. Their estimates, ranging from 2.34 per cent to 4.70 per cent and averaging 3.64 per cent are distinctly higher and more variable than ours made with the unmodified Folin Marenz method. Their modification was to precipitate the protein with trichlor

with 6N H_2SO_4 substances interfering with the estimation of cystine by the Folin-Marenzi method may possibly be formed

It was thought that an estimate of the number of S-S groups in denatured globin by reducing them with thioglycolic acid and estimating the SH groups formed, might serve as a check on the cystine estimation because in some denatured proteins the number of S-S groups is equivalent to the cystine content. Unfortunately estimations of the S-S groups of denatured globin yield results that are both so variable and so high (over 1 per cent) that it appears doubtful whether the method of estimation is applicable to globin. It is possible that thioglycolic acid remains adsorbed to the protein. In the absence of confirmatory evidence it is unlikely that estimation of the cystine content of globin by our method is correct.

Finally, it should be stated that the difficulties encountered in estimating the cystine content of globin do not affect the results reported in this paper, for confidence can be placed in our estimate of the cysteine content of globin. In this case estimation of SH groups serves as a check, since it is found that the number of SH groups in denatured globin is equivalent to the cysteine content of hydrolyzed globin.

Hemoglobin Reaction between Hemoglobin and Ferricyanide

The reagents used were a 10 per cent solution of horse oxyhemoglobin prepared by Heidelberger's method, M/2 potassium ferricyanide, 3.4 M KH_2PO_4 - K_2HPO_4 pH 6.8, M/2 KH_2PO_4 - K_2HPO_4 pH 7.3, M/1 K_2HPO_4 , M/2 H_3BO_3 -NaOH pH 9.0, and M/2 H_3BO_3 -NaOH pH 9.6. 5 cc hemoglobin solution were mixed with 10 cc buffer and 3 cc ferricyanide, and the mixture allowed to stand at room temperature for 30 minutes. Under these conditions all the SH groups of denatured globin react with ferricyanide. 8 cc of the pH 6.8 buffer were then added, and the solution was dialyzed against distilled water in a rocking dialyser for 20 hours to remove the ferricyanide. After adding the various buffer solutions to hemoglobin the pH of the resulting mixtures was measured with the glass electrode, with the following results: after adding the pH 7.30 buffer, the pH of the mixture was 7.30, after adding K_2HPO_4 the pH was 8.75, after adding the pH 9.00 buffer, the pH was 8.94, and after adding the pH 9.6 buffer, the pH was 9.5.

acetic acid and hydrolyze the precipitated protein with 14N H_2SO_4 instead of drying the protein and hydrolyzing with 6N H_2SO_4 . Following their procedure we obtained, in agreement with them, a cystine content of 3.44 per cent. The final concentration of H_2SO_4 in the hydrolysate was about 11.3N. The modification of the Folin-Marenzi method introduced by Tuchman, Reiner, and Sobotka accounts for the high values they obtained, and it probably also accounts for the variability of their results, for it is unlikely that the concentration of H_2SO_4 used by them for hydrolysis was kept constant.

Reaction between Hemoglobin and Cystine

A concentrated cystine solution was prepared by adding to 0.75 gm. cystine $N/2$ KOH (about 12.5 cc.) until practically all the cystine dissolved, but not enough alkali to make the pH exceed 9.6. The solution was blue to thymol blue but colorless to thymolphthalein. To this were added 5 cc. of a 10 per cent solution of horse carbon monoxide hemoglobin. Carbon monoxide was bubbled through the solution, the flask was then stoppered and allowed to stand in the dark for $1\frac{1}{2}$ hours when 10 cc. of 3.4×10^{-2} M pH 6.8 KH_2PO_4 buffer were added. The precipitated cystine was removed by centrifuging.

Preparation of Globin—The acid acetone method was used to prepare globin from hemoglobin that had been treated with ferricyanide or cystine. In preparing globin from hemoglobin that had been treated with cystine, it was necessary to add more acid than is usually employed because of the phosphate buffer present. To the hemoglobin solution were added 10 cc. N HCl and to the 600 cc. of acetone used another 10 cc. N HCl were added. Removal of heme made it possible to estimate the SH groups of hemoglobin by the methods used for other proteins. The globin precipitated by acetone was not separated by filtration but by centrifuging. This was done in a 250 cc. centrifuge flask, and the globin was washed free of pigment by further additions of acid acetone. Most of the acetone was removed by centrifuging and the rest was removed by washing several times with 5 per cent trichloroacetic acid.

Estimation of SH Groups of Untreated and Oxidized Globin—The SH groups of the various preparations of globin were estimated by the 'direct' method (Paper I). Globin was mixed with a cystine solution and the quantity of cystine formed was equivalent to the number of SH groups of the protein. The number of active SH groups of any given sample of hemoglobin was equal to the difference between the number of SH groups in globin prepared from untreated hemoglobin and the number in globin prepared from hemoglobin that had reacted with ferricyanide or cystine.

Reversal of pH Effect—To 5 cc. of the hemoglobin solution were added 10 cc. M KH_2PO_4 and after 30 minutes 6 cc. 3.4×10^{-2} M KH_2PO_4 buffer, pH 6.8. This globin was treated with ferricyanide and then dialyzed as described above.

The cystine content (SH groups of hydrolyzed globin) was estimated by the method described by Mirsky and Anson (Paper I).

Reaction of Denatured Globin with Ferricyanide

0.5 gm. of denatured globin in the form of a dry powder prepared by the acid acetone method (Anson and Mirsky, 1929-30) was dissolved in 50 cc. of water. This was diluted with water to a volume of 200 cc. and to the solution were added 15 cc. of concentrated trichloroacetic acid (trichloroacetic acid dissolved in an equal weight of water). The mixture was centrifuged, the supernatant fluid discarded and the precipitate transferred to a 50 cc. centrifuge tube. In this it was well stirred with 40 cc. of a 20 per cent sodium sulfate solution and 10 cc. of a 1.2

M pH 7.3 KH_2PO_4 - K_2HPO_4 buffer The suspension was centrifuged and the precipitate was mixed with 40 cc sodium sulfate solution and 5 cc 3.4 M KH_2PO_4 - K_2HPO_4 pH 6.8 buffer After centrifuging, the protein was suspended in a mixture of 25 cc sodium sulfate solution, 3 cc pH 6.8 buffer, and 4 cc M/2 potassium ferricyanide This tube stood for an hour with occasional agitation and was then centrifuged The globin was washed with a 10 per cent solution of sodium sulfate by repeated mixing and centrifuging until no ferricyanide could be seen in the washings The SH groups of this protein were estimated by the direct method, by the quantity of cysteine formed when the protein was mixed with a cystine solution (Paper I) Another sample was hydrolyzed, and the cysteine content of the hydrolysate estimated

Experiments with the Lens Proteins

Lenses dissected from eyes of oxen were thoroughly mashed in a mortar During the mashing small amounts of physiological saline were added so that a thin, homogeneous, paste-like mixture of the proteins was prepared One portion was denatured with trichloroacetic acid and then treated with iodoacetate to estimate the SH groups of the denatured proteins (Paper I) Other portions, while in the native state, were mixed with buffers varying in pH from 6.8 to 9.2 and were then treated with iodoacetate The buffers, all M/2, were phosphate at 6.8, 7.2, 7.6, and 8.0 and borate at pH 8.6 and 9.2 To approximately 7 cc of a protein mixture (containing about 600 mg of protein) were added 66 cc buffer solution and 33 cc M/10 iodoacetate (iodoacetic acid neutralized with sodium hydroxide) From this point the procedure was the same as that of the "indirect" method for estimating protein SH groups (Paper I) The quantity of cysteine found in the hydrolysate of such a preparation was equivalent to the number of SH groups of the native protein that were *not* active at a given pH By subtracting the number of inactive groups from the total number present, the number of active groups was obtained

EXPERIMENTAL RESULTS

SH groups are recorded in terms of cysteine, that is as the quantity of cysteine which would have the same sulfur content, the amount of cysteine being expressed as per cent of the total amount of protein

Globin and Hemoglobin

1 Cysteine content of hydrolyzed horse globin—0.42 per cent
This is equivalent to 2 molecules of cysteine per molecule of hemoglobin containing 4 iron atoms

2 SH groups of denatured globin—0.38 per cent

3 Cysteine content of hydrolyzed globin after denatured globin had been treated with potassium ferricyanide at pH 6.8—Nil

4 SH groups of denatured globin prepared from hemoglobin treated with potassium ferricyanide at

(a) pH 6.8 —0.36 per cent

(b) pH 7.3 —0.28 per cent

(c) pH 8.96—0.22 per cent

(d) pH 9.5 —0.15 per cent

5 SH groups of native hemoglobin at

(a) pH 6.8 —(2) minus (4a)—0.02 per cent

(b) pH 7.3 —(2) minus (4b)—0.10 per cent

(c) pH 8.96—(2) minus (4c)—0.16 per cent

(d) pH 9.5 —(2) minus (4d)—0.23 per cent

6 SH groups of globin prepared from hemoglobin brought to pH 8.75 for 15 minutes and then treated with potassium ferricyanide at pH 6.8—0.35 per cent

7 SH groups of globin prepared from carbon monoxide hemoglobin treated with cystine at approximately pH 9.6 (without methemoglobin formation in contrast to the experiments with ferricyanide)—0.14 per cent

Proteins of the Crystalline Lens

1 Cysteine content of the protein hydrolysate—1.25 per cent

2 Cysteine content of the protein hydrolysate after denatured protein had been treated with iodoacetate at pH 7.0—Nil

3 SH groups of denatured protein (1) minus (2)—1.25 per cent

4 Cysteine content of protein hydrolysate after the native protein had been treated with iodoacetate at

(a) pH 6.8—0.97 per cent

(b) pH 7.2—0.89 per cent

(c) pH 7.6—0.79 per cent

(d) pH 8.0—0.71 per cent

(e) pH 8.6—0.65 per cent

(f) pH 9.2—0.52 per cent

5 Active SH groups of native protein at

(a) pH 6.8 (3) minus (4a)—	0.28 per cent
(b) pH 7.2	0.36 per cent
(c) pH 7.6	0.46 per cent
(d) pH 8.0	0.54 per cent
(e) pH 8.6	0.60 per cent
(f) pH 9.2	0.73 per cent

6 Native proteins having been at pH 9.4 for 2 hours in the absence of oxygen treated with iodoacetate at pH 7.2—cysteine content of protein hydrolysate—0.90 per cent

SUMMARY

Hemoglobin and the proteins of the crystalline lens contain active SH groups while in the native state, the number of active groups increasing as the pH rises. All the SH groups of denatured globin and of the denatured lens proteins are active at a pH so low that practically none of the SH groups of native hemoglobin and of native lens protein are active. The effect of denaturation on the SH groups of a protein is to extend towards the acid side the pH range of their activity.

It is possible to oxidize the iron-porphyrin and the SH groups of hemoglobin independently of each other.

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THE REDUCING GROUPS OF PROTEINS

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In the chemistry of proteins much is known about their acid base groups but little about other groups. Sulfhydryl and disulfide groups are the only other ones which have been studied with some care, and their importance in investigations of enzymatic activity (Sumner, Lloyd, and Poland, 1933) and protein denaturation (Mirsky and Anson 1935-36a) is now recognized. In this paper the existence of other reducing groups in addition to the SH groups is demonstrated and some of their properties are briefly described.

In a previous paper it was shown that ferricyanide can oxidize the SH groups of proteins (Mirsky and Anson, 1934-35). In this paper it will be shown that ferricyanide can also oxidize other groups, that these other groups probably belong to tyrosine and tryptophane, and that the activity of these other groups, as of the SH groups, increases with increase of pH, rise in temperature, and with denaturation of the protein. Whereas the oxidation of SH to S-S by ferricyanide is a definite reaction under suitable conditions— $2 \text{ SH} + 2 \text{ ferricyanide} = 1 \text{ S-S} + 2 \text{ ferrocyanide}$ —the reaction of the other groups with ferricyanide is not so definite. The greater the ferricyanide concentration and the longer the time of reaction, the more oxidation by ferricyanide takes place. Finally the groups other than the SH groups which are oxidized by ferricyanide are weaker reductants than the SH groups. Conditions can in some cases be found under which they are not oxidized by ferricyanide when under the same conditions protein SH groups are oxidized, and cystine does not oxidize the other groups whereas it can oxidize SH groups.

Knowledge of the new reducing groups is significant in the study of protein denaturation and of the reducing properties of tissues. It can

now be seen that the activation of SH and S-S groups in protein denaturation is part of a more general process. The discovery of the activation of these new reducing groups suggests that still other groups may be activated by denaturation. These reducing groups possess some activity even when a protein is in the native state. The reducing property of the tissue proteins is probably important in maintaining that "reducing environment" which is such a striking characteristic of the interior of the cell.

Method of Detection —The new reducing groups can be most clearly detected in proteins which contain no SH groups, such as zein and serum globulin. The evidence that reducing groups are present is that when potassium ferricyanide is added to these proteins ferrocyanide is formed. Since ferrocyanide can be estimated as prussian blue, the method is simple, sensitive, and exact. In proteins containing SH groups other reducing groups may be observed if the former are first oxidized. For this purpose a mild oxidant which reacts only with the strongly reducing SH groups is required. Cystine possesses this property (Mirsky and Anson, 1934-35) and by using it to oxidize the SH groups of the muscle proteins, egg albumin and globin, and then treating the proteins with ferricyanide, the other reducing groups of these proteins have been studied.

Factors Affecting the Reducing Groups of Proteins —The activity of reducing groups depends on whether a protein is in the native or denatured state. In the case of egg albumin this is most clearly observed. Native egg albumin at pH 9.6 does not reduce ferricyanide at all, and since the test for ferrocyanide is highly sensitive even a trace of reducing activity would be detected. Denatured egg albumin, the SH groups of which have been oxidized by cystine readily reduces ferricyanide at pH 9.6. In other proteins the difference between the native and denatured state is not so marked. Native edestin reduces ferricyanide, denatured edestin reduces it twice as rapidly. Muscle proteins which have not been exposed to a denaturing agent reduce ferricyanide almost as readily as do those which have been denatured by trichloroacetic acid.

Reducing activity of both native and denatured proteins depends upon the pH, the higher the pH the greater is their activity. The effect of denaturation is to shift the pH range of activity so that a

protein is able to reduce in a more acid medium than it could while in the native state. Reducing activity is also enhanced by a rise in temperature. In edestin a rise in temperature of 10° increases the activity about 45 per cent. Reducing activity varies from one protein to another. Denatured edestin, for instance, is about six times as active as denatured serum globulin, but not quite as active as zein. As already mentioned, the extent of increase in activity on denaturation is a characteristic of each protein.

All these factors—denaturation, hydrogen ion concentration, temperature, nature of the protein—have also been found¹ to influence the activity of protein SH groups. In other ways, now to be described, properties of SH and of other reducing groups differ markedly. Although SH groups are easily oxidized beyond the S-S state, it is possible with ferricyanide (and also with other oxidants) to control conditions so that a clearly defined reaction occurs in which two molecules of ferricyanide react with two SH groups with the formation of two molecules of ferrocyanide and one S-S group. This occurs when denatured globin reacts with ferricyanide at pH 6.8 (Mirsky and Anson, 1935-36*b*). When ferricyanide oxidizes the other reducing groups of a protein the reaction is not clearly defined, the quantity of ferricyanide reduced depends on the concentration used and on the time during which it is in contact with protein. The extent of reduction depends on the concentration of ferricyanide even when the amount of ferricyanide used is 30 to 50 times in excess of the reducing capacity of the protein. A fivefold increase in concentration of ferricyanide may cause a two and-one half fold increase in quantity of ferricyanide reduced. As protein stands in contact with an excess of ferricyanide, the amount of ferrocyanide formed continually increases, although the quantity formed per hour gradually decreases. The limit of the reducing capacity of the protein is not reached even after 24 hours. (In periods as long as this some hydrolysis of protein may occur.)

In the muscle proteins presence of SH groups appears to increase the activity of the other reducing groups. If the SH groups of denatured proteins of muscle are oxidized by cystine and the ability of

¹ In native proteins activity of SH groups increases with a rise in temperature (unpublished experiments)

these proteins to reduce ferricyanide compared with that of proteins still retaining their SH groups, it is found that the latter reduce three times more ferricyanide in the same time. It should be noted that since much of the excess of cystine used to oxidize the SH groups remains adsorbed to the protein, this cystine may in some way affect the other reducing groups.

Comparison of Reducing Capacity and Intensity of SH Groups and of the New Groups

The reducing capacity of these groups in the muscle proteins is more than twice that of their SH groups. On the other hand, the reducing intensity of SH groups is much greater, for they unlike other reducing groups of protein, reduce cystine and phosphotungstate. In denatured globin the difference in reducing intensity of the various groups can be observed in their reactions with the same reagent—ferricyanide. The reducing activity of all these groups increases as the medium is made more alkaline. At pH 6.8 all the SH groups, and only these groups, of denatured globin react with ferricyanide. As the pH rises, other reducing groups become detectable, and at pH 9.6 are quite active.

Identification of the Groups—Among the known amino acids of proteins those which might possibly be responsible for the reducing properties described above are histidine, tyrosine, and tryptophane. Tyrosine and tryptophane reduce ferricyanide, histidine does not. In the reduction of ferricyanide the general behavior of tyrosine and tryptophane resembles that of proteins. When tyrosine and ferricyanide are mixed, reduction continues for at least 5 hours. The reducing activity increases with a rise of temperature, of pH, or in the concentration of ferricyanide. At 37° 1 molecule of tyrosine can reduce at least 2.6 molecules of ferricyanide. These facts suggest that tyrosine and tryptophane provide the reducing groups of proteins. This conclusion is supported by a study of the reducing properties of zein and gelatin. Although zein contains no tryptophane, it contains much tyrosine, and it actively reduces ferricyanide. A preparation of gelatin containing very little tyrosine reduces only about one-twenty-fifth as much ferricyanide as does an equal weight of edestin under the same conditions.

EXPERIMENTAL

Reagents Used *Proteins*—The egg albumin used had been recrystallized three times. The serum globulin, prepared from horse serum by half saturation with ammonium sulfate, was a mixture of eu and pseudoglobulin. The globulin had been freed of albumin by reprecipitation. Zein was prepared by the method of Osborne and Harris. Crystalline edestin was purchased from Hoffmann La Roche. Mixed proteins of rabbit muscle were prepared by washing finely minced muscle six times with water, the muscle suspended in 20 times its volume of water was stirred for 5 minutes and the water was then decanted. The mixed proteins of frog muscle were prepared in the denatured state (Mirsky and Anson, 1934-35). Globin was prepared by the acid acetone procedure (Anson and Mirsky).

The potassium ferricyanide used contained no ferrocyanide. Tungstic acid was prepared by adding to 20 cc. of a 10 per cent sodium tungstate solution 960 cc. water and then 20 cc. of $\frac{3}{4}$ N H₂SO₄. A solution of ferric sulfate in gum ghatti was prepared as described by Folin and Malmros.

Procedure—To several cc. of the protein preparation in a graduated centrifuge tube were added 1 cc. of a buffer solution and several tenths of a cc. $\frac{M}{2}$ potassium ferricyanide. When the mixture had stood for a definite time 10 cc. tungstic acid and 0.5 cc. N H₂SO₄ were added and the suspension centrifuged. After the total volume was recorded a measured portion of the supernatant fluid was removed and diluted to 20 cc. with water. To this were added 5 cc. of the Fe SO₄ gum ghatti solution. After 3 minutes the intensity of the blue color formed was measured in a colorimeter. A blue glass served as a standard which matched prussian blue in the fairly monochromatic light transmitted by a suitable red filter. The standard glass was calibrated by comparison with the prussian blue formed when 0.8 mg. of ferrocyanide was added to the Fe₂SO₄ solution.

The oxidation of proteins by cystine has been described (Mirsky and Anson 1934-35).

Effect of Denaturation

1 Qualitative Test on Egg Albumin—To 4 cc. native egg albumin containing 150 mg. protein were added 10 cc. $\frac{M}{2}$ pH 9.6 borate buffer and 0.3 cc. $\frac{M}{2}$ potassium ferricyanide. The mixture stood for 30 minutes at 37°C. The same quantity of denatured egg albumin coagulated by heat, and then oxidized with cystine was treated in the same manner. On subsequent addition of ferric sulfate no prussian blue was formed in the solution which had been in contact with native egg albumin; an intense prussian blue was obtained in the other solution.

2 Edestin—Denatured edestin was prepared by adding to 200 cc. of a 1 per cent solution of edestin, 20 cc. of a 50 per cent solution of trichloroacetic acid. After centrifuging and decanting the supernatant fluid, the denatured edestin was washed with water and trichloroacetic acid, dehydrated with acid acetone, and dried (Mirsky and Anson 1934-35). This powder was dissolved in a half saturated urea solution to 1.2 cc. of which (containing 24 mg. edestin) were added 0.3 cc.

saturated solution of urea, 1 cc M/2 pH 9.6 borate buffer, and 0.25 cc M/2 potassium ferricyanide. A solution of native edestin was prepared, 2.5 cc of which contained 24 mg protein and the equivalent of 1 cc of the borate buffer. To 2.5 cc of this solution was added 0.25 cc M/2 potassium ferricyanide. Both solutions were allowed to stand for 15 minutes at 20°, when to each were added 7 cc water, 0.5 cc N H₂SO₄, and 10 cc tungstic acid. In the solution of denatured edestin, 1.05 mg ferrocyanide were formed, in the solution of native edestin 0.504 mg was formed.

3 Proteins of Rabbit Muscle—Part of the washed muscle was denatured by trichloroacetic acid, after which the tissue was washed free of acid (Mirsky and Anson, 1934-35). 5 cc of this suspension and 5 cc of tissue which had not been treated with trichloroacetic acid were used. To each were added 2 cc M/2 pH 9.6 borate buffer and 0.7 cc M/2 potassium ferricyanide. The mixtures were allowed to stand with frequent agitation at room temperature for 1 hour. In each tube were then placed 1 cc N H₂SO₄ and 20 cc of tungstic acid. At the end of the experiment the dry weight of tissue which had been treated with trichloroacetic acid was found to be 220 mg, the weight of the other tissue was 228 mg. The former produced 5.56 mg ferrocyanide, the latter 4.65 mg. It has been found (unpublished experiments) that at pH 9.4 the proteins of muscle are not denatured.

Effects of pH and Temperature—1.0 gm edestin was dissolved in 19.5 cc water plus 0.5 cc N HCl. To 2 cc (containing 100 mg protein) in a 50 cc centrifuge tube were added 45 cc water and then 1 cc of 50 per cent trichloroacetic acid. After centrifuging the precipitate was suspended in 45 cc of a 2 per cent sodium sulfate solution. This suspension was centrifuged and the supernatant fluid was decanted. Such a preparation of edestin was used in each of the experiments to be described. The protein was mixed with 20 cc of water, 20 cc of a 20 per cent sodium sulfate solution, 4 cc M/2 borate buffer, stirred, and then centrifuged. To the precipitate were added 3 cc water, 1 cc buffer, and 0.2 cc M/2 potassium ferricyanide. This mixture stood for 30 minutes, when 15 cc of tungstic acid and 0.5 cc N H₂SO₄ were added. The amount of ferrocyanide formed was estimated. At 27° 0.915 mg ferrocyanide was formed at pH 9.6, 0.65 mg at pH 9.0, and 0.59 mg at pH 8.4. At 37° 1.34 mg were formed at pH 9.6, 0.96 mg at pH 9.0, and 0.77 mg at pH 8.4.

Activities of Edestin, Serum Globulin, Zein, and Gelatin Compared

Denatured globulin was prepared by dissolving a little of the ammonium sulfate precipitate in 35 cc of water, and placing the tube in a water bath, heated to 90°. When coagulation was practically complete, the tube was cooled and centrifuged. The precipitate was washed with a mixture of sodium sulfate and pH 9.6 borate buffer, and subsequently treated with ferricyanide, as in the experiments on edestin. At the end of the experiment the dry weight of the protein was estimated. 100 mg zein were dissolved in 5 cc of water plus 0.5 cc N/2 NaOH. To the solution were added 2 cc M/2 pH 9.6 borate, 0.25 cc N HCl (when zein precipitated) and 0.25 cc M/2 potassium ferricyanide. Reduction

proceeded for 30 minutes at 27°. Under these conditions 306 mg serum globulin formed 0.49 mg ferrocyanide, 100 mg zein 1.34 mg ferrocyanide, and 100 mg edestin 0.195 mg ferrocyanide. To compare gelatin with denatured edestin the latter must be dissolved in urea, as previously described. 120 mg gelatin were dissolved in 3 cc. of water to which were added 1 cc. $M/2$ pH 9.6 borate buffer and 0.3 cc. $M/2$ potassium ferricyanide. In 30 minutes, at room temperature, 0.33 mg ferrocyanide was formed. Under the same conditions about one sixth (21 mg) of the quantity of edestin formed 1.4 mg of ferrocyanide.

Effects of Time and Concentration of Ferricyanide

In experiments on denatured edestin dissolved in urea as described above, at 25° 21 mg edestin when mixed with 0.25 cc $M/2$ potassium ferricyanide formed 0.99 mg ferrocyanide in 15 minutes and 1.4 mg in 30 minutes. When only 0.1 cc of ferricyanide was used, 0.56 mg ferrocyanide was formed in 30 minutes and with 0.5 cc ferricyanide 1.83 mg ferrocyanide were formed.

100 mg precipitated denatured edestin were treated with ferricyanide at pH 9.6 as described above. After the mixture had stood at 37° for 24 hours, 40 cc of water in 2 cc of a pH 6.8 $3.4 M$ KH_2PO_4 K_2HPO_4 buffer were added. The suspension was centrifuged. The precipitate was washed in a 2 per cent sodium sulfate solution, by being repeatedly suspended and then centrifuged, until no yellow pigment remained. The edestin was then again mixed with borate and ferricyanide and allowed to remain at 37° for 30 minutes. During this period 0.72 mg ferrocyanide was formed. Under these conditions, freshly prepared denatured edestin formed 1.34 mg ferrocyanide.

Effect of Presence of SH Groups on Activity of Other Reducing Groups

Minced frog muscle was thoroughly washed with trichloroacetic acid (Mirsky and Anson, 1934-35) to remove soluble reducing substances. The cysteine content of one portion was estimated. Another portion was oxidized with cystine. The reducing activity of this sample was compared with that of another sample which still contained SH groups. Both samples were transferred to 50 cc centrifuge tubes and there washed several times with water. Each preparation was then suspended in 40 cc. of a 10 per cent sodium sulfate solution plus 5 cc. $M/2$ pH 9.6 borate buffer. After centrifuging to each precipitate were added 2 cc. of water, 1 cc buffer and 0.5 cc. $M/2$ potassium ferricyanide. The mixtures were allowed to stand with occasional agitation at 20° for 1 hour. In the tube containing muscle oxidized by cystine 3.32 mg ferrocyanide were formed and the dry weight of the protein was 644 mg. This is equivalent to 0.516 mg ferrocyanide for 100 mg of protein. In the other tube 5.96 mg ferrocyanide were formed and the dry weight was 231 mg—2.58 mg ferrocyanide for 100 mg of protein. From this figure must be subtracted 1.00 mg ferrocyanide which was formed by the SH groups of the proteins (the cysteine content of which is 0.58 per cent). This leaves 1.58 mg ferrocyanide as having been formed by the non SH reducing

groups of the proteins containing SH groups, as against 0.516 mg ferrocyanide by those groups in the proteins with oxidized SH groups²

Reducing Properties of Tyrosine and Tryptophane

100 mg tyrosine were dissolved in 98 cc of water plus 2 cc M/2 pH 9.8 borate buffer and 100 mg tryptophane were dissolved in 100 cc of water. In all experiments 0.5 cc tyrosine or 1 cc of tryptophane solution were mixed with 1 cc M/2 borate buffer and (unless otherwise stated) 0.2 cc M/2 potassium ferricyanide. After a definite interval of time 17 cc of water, 0.5 cc N H₂SO₄, and 5 cc of ferric sulfate-gum ghatti were added. At pH 9.6 and at room temperature tryptophane formed 1.21 mg ferrocyanide in 30 minutes, at pH 8.4, 0.272 mg was formed. Under these conditions tyrosine formed 1.02 mg at pH 9.6 and 0.745 mg at pH 9.0. Tryptophane at pH 9.6 formed 0.48 mg ferrocyanide in 2 minutes, 1.21 mg in 30 minutes, 1.73 mg in 60 minutes, and 2.68 mg in 120 minutes. Tyrosine at pH 9.0 formed 0.38 mg ferrocyanide in 5 minutes, 0.57 mg in 25 minutes, 0.82 mg in 70 minutes, 1.01 mg in 190 minutes, and 1.44 mg in 3000 minutes. Tryptophane at pH 8.4 in presence of 0.4 cc M/2 potassium ferricyanide formed 0.53 mg ferrocyanide in 50 minutes at 26°, at 36° it formed 0.99 mg. On the other hand, at 16° and at 23°, tyrosine formed about the same quantity of ferrocyanide. Tyrosine at 31° and pH 9.6 in presence of 2.0 cc of M/2 potassium ferricyanide formed 1.25 mg ferrocyanide whereas in presence of 2 cc of M/20 potassium ferricyanide it formed 0.24 mg ferrocyanide.

SUMMARY

1 Intact, unhydrolyzed proteins possess in addition to SH groups other reducing groups which can be oxidized by ferricyanide.

2 The activity of these reducing groups, like that of SH groups, is enhanced by denaturation of the protein and by increase of pH and temperature.

3 These groups differ from SH groups in the manner in which their activity is dependent on concentration of ferricyanide and time of contact with ferricyanide.

4 The activity of these groups is increased if protein SH groups are present.

5 The number and activity of these groups varies from protein to protein.

6 These groups are probably contained in the tyrosine and tryptophane components of proteins.

² This experiment should be repeated with muscle proteins carefully freed of lipoids. Adhering lipoids may be responsible for some of the effects observed.

7 The significance of these reducing groups for an understanding of protein denaturation and the reducing properties of tissues is indicated

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A KINETIC ANALYSIS OF THE ENDOGENOUS RESPIRATION OF BAKERS' YEAST

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I

We will discuss in this paper the kinetics of endogenous respiration of bakers' yeast, that is, the respiration of intact yeast cells suspended in non nutrient media. In succeeding papers results growing from an examination of the nature of the metabolic processes involved will be presented. The purpose of this series is to demonstrate the necessity of describing the behavior of metabolic systems at their native loci by the use of *in vivo* procedures. By dealing with experimentally verifiable rate controlling steps in the various metabolic systems we will show how these complex chains of reactions may behave as distinct functional units at one time and, depending upon the imposed experimental conditions, as interrelated but yet coordinated systems. Since the overall metabolic activity can be shown to be governed by these rate controlling "loci,"¹ integrated action of the myriads of simultaneous reactions is achieved within a cell. It is felt that this method of investigation is a necessary procedure which should precede any final application of the results of *in vitro* studies of enzyme action to the dynamical organization of *in vivo* metabolic processes.

As a general rule in plant cells and tissues the rate of respiration under starvation conditions sooner or later decreases with time after the stored material has been reduced below a certain "critical concentra-

¹ Obviously in all but 'zero order reactions' (for an example cf p 471) the instantaneous rate of respiration may be a function of the concentration of reacting materials. The rate constant in these cases is then the appropriate constant describing the activity of the pace setting locus e.g., a first order constant. We imply that the behavior of the descriptive constant for the rate of decomposition of a relatively large amount of substrate reflects the physical and chemical characteristics of this locus, whatever its real nature may be.

tion" This has been shown in *Aspergillus niger* by Kosinski (1902) and in yeast most recently by Geiger-Huber (1934) It is assumed that bacteria maintain themselves in non-nutrient solutions by decomposition of their internal stores (Rahn, 1932)

The term "autofermentation" has been applied to the decomposition of internally stored carbohydrates or their products of hydrolysis in yeast juices, dried yeast, pressed yeast, and intact yeast (*cf* Harden, 1932, von Euler and Lindner, 1915) Harden and Young (1902) showed that glycogen is the principal carbohydrate reserve of yeast and it is generally held that the ultimate substrate in autofermentation is glycogen (*cf* Harden, 1932,² Warburg, 1927,³ Meyerhof, 1925, Warkany, 1924)

The tacit assumption has been that autofermentation proceeded by the same general mechanism as the respiration and fermentation of sugar added to the medium The work which we are to describe emphasizes the necessity for exercising great care in the use of the term autofermentation The metabolic utilization of reserve substances exhibits such differences in intact living yeast as compared with variously treated preparations, that the term autofermentation is not strictly applicable to the former For reasons which will be given in another paper we prefer the term *endogenous respiration* and will reserve autofermentation for those cases in which the metabolism can be shown to be of a truly fermentative type as is evidently the case with yeast juice and pressed yeast

II

EXPERIMENTAL PROCEDURES

Two strains of bakers' yeast, *Saccharomyces cerevisiae*, were employed, one was a pure strain grown in the laboratory, the other a pure strain grown in large quantities under commercial conditions and obtained from the Fleischmann Company through the kindness of Dr Charles N Frey This latter strain is essentially commercial bakers' yeast, but is handled under such conditions as to exclude almost all possible contaminants The time at which each lot was separated from its culture medium was noted on each package, the yeast was

² Pp 33, 34, 40, 143, 161, and 186

³ P 364

kept under refrigeration en route from the factory to our Laboratory. This strain will be referred to as GM yeast. The pure strain which we cultivated was one already employed in connection with several studies made in this Laboratory (Richards, 1928, Oster, 1934-35). It was *Saccharomyces cerevisiae* Hansen, obtainable from the American Type Culture Collection, Chicago, formerly No. 2335, and now re-numbered No. 4360. We will refer to this strain as Strain 4360.

Stock cultures of Strain 4360 were maintained at low temperature (7 C) on agar slants made up with 1.5 per cent malt extract broth (Difco) and 2 per cent agar sterilized 20 minutes at 15 lbs. pressure. At least 2 weeks prior to use the yeast was transferred to Williams' medium (*cf.* Williams 1920, Williams, Wilson, and von der Ahe 1927) and sub-cultures made every 2-3 days. Pure culture methods were used at all times. During any one period of experimentation stock cultures were maintained in Williams' medium.

The actual experimental material was incubated for the desired time in glass towers 30 × 3.5 cm. at 25-26 C. These had a tube sealed in at the top reaching to within ½ cm. of the bottom to allow aeration from a purified compressed air supply. Aeration is necessary, for as shown by Stier, Arnold and Stannard (1933-34) large 'clumps' of yeast interfere with proper measurement of the turbidity of the suspension by the photoelectric densitometer, and probably some what with measurement of the metabolism as well. The aeration was sufficiently vigorous to reduce the number of clumps containing over 4 cells to less than 5 per cent of the total number of cells.

In the earlier portion of the work we used transfers from the original stock culture obtained from the American Type Culture Collection. At a later date single cells from this culture were isolated by a dilution technique employing Petri dishes and malt extract agar. The loci of single cells were marked after examination under the binocular microscope and several colonies arising from such single cells were separately inoculated onto agar slants and then later into Williams' medium. The most successful one of these was selected for future work. We repeated several experiments on this newly isolated strain and compared its behavior with that of our original pure line. No differences in respiratory activity or in its metabolic behavior could be detected.

The yeast was centrifuged away from the culture medium at approximately 3000 R.P.M. for 3-5 minutes. Fresh sterile M/15 KH_2PO_4 solution (pH 4.5), or rarely Sørensen phosphate buffer at a series of pH values, was added; the cells were washed, recentrifuged and resuspended at the proper concentration in the new medium. One washing was sufficient to remove the last traces of the culture medium but not enough to wash out appreciably any of the soluble components of the cell.

The GM yeast was stored in a refrigerator at 7 C and was used within 24-48 hours after its arrival. For each experiment a portion was removed from the

center of the package in a sterile transfer cabinet and made up to the desired concentration with sterile M/15 KH_2PO_4 , usually after one centrifugal washing

The photoelectric densitometer (Stier, Arnold, and Stannard, 1933-34) was used as a quick and accurate method of obtaining yeast suspensions of uniform and known concentration for any series of experiments. The densitometer was calibrated in terms of dry weight of yeast. The density of the suspensions used for the experiments here reported was made high since the rate of endogenous respiration of intact yeast is only 0.4-0.05 of that of yeast suspended in 2 per cent dextrose-phosphate solution (*cf* Warburg, 1927, Meyerhof and Iwasaki, 1930)

The oxygen consumption and aerobic CO_2 production were measured in Warburg respirometers. The CO_2 production was measured by the two vessel method discussed by French, Kohn, and Tang (1934-35). The shaker speed necessary to insure freedom from the limits set by diffusion was ca 120 complete oscillations per minute with an amplitude of 8 cm. As the rate of respiration declined the time between readings was increased so that the experimental error of reading manometer deflections was held to within ± 2.5 per cent. Inaccuracies due to the retention of CO_2 by Na_2HPO_4 when this substance is used in preparing buffer solutions for manometric measurements of metabolism were reduced to a minimum by using only KH_2PO_4 , as recommended by Krebs (1928). Addition of acid at the end of an experiment was thus unnecessary.

The growth of the yeast respiring in sterile M/15 KH_2PO_4 solution was determined by means of the densitometer. Two samples were prepared, one at the original concentration of yeast and another diluted with sterile primary phosphate solution to a concentration sufficiently low to eliminate any growth inhibition due to crowding (*cf* Stephenson, 1930⁴). The samples were kept in densitometer tubes which were immersed in a thermostat. No growth was ever observed in pure phosphate solutions. The number of cells taking up methylene blue at the beginning and end of the experiment was found to be low and constant (*cf* Richards, 1932, for details regarding the significance of this method of estimating the number of "dead" cells). Care was exercised to maintain reasonable sterility during all phases of an experiment. Microscopic examination after Gram staining revealed no bacterial growth even in experiments lasting 48 hours.

All (or representative) samples from the Warburg vessels were tested for change in pH by two methods, indicator and quinhydrone electrode with calomel half-cell, and found to remain constant within 0.1 pH unit during 24 hours time.

III

The Rate of Endogenous Respiration as a Function of Time in a Non-Nutrient Medium

Few studies of yeast under starvation conditions do more than state that the rate of O_2 consumption or CO_2 production declines with time

Geiger-Huber (1934) in a recent paper figures both differential and integral curves for the oxygen consumption of Delft beer yeast suspended in $M/15$ KH_2PO_4 solution showing an initial rapid decline in rate to a constant level which persisted for the duration of the experiment, but he does not give a kinetic analysis of his data

If bakers' yeast is suspended in a pure phosphate buffer medium the subsequent rate of respiratory exchange is a function of both the initial age of the cells and the time in the medium. If a young (24 hour) culture is used, the rate of respiration declines with time from

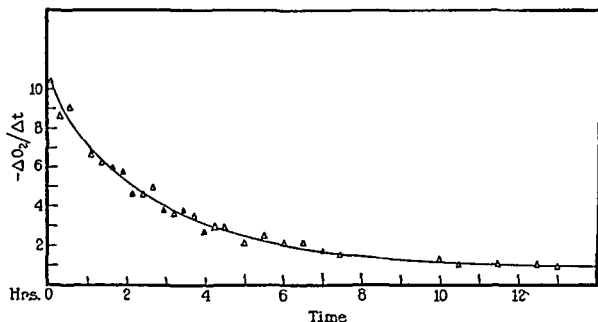


FIG 1 The rate of O_2 consumption (c.mm / 10 min) as a function of time for a 16 hour culture suspended in $M/15$ KH_2PO_4 solution at pH 4.5 (Experiment at 25 C. Strain 4360 cultured at 25 C)

the first point while in older cultures a varying period of constant rate precedes the decline, which is somewhat slower in this case. For convenience we have termed these rate time relations the "dissimilation curve". The events occurring in young cultures will be discussed first, followed by a short account of the influence of the age of the cell on the course of the dissimilation curve.

Young Cultures

Fig 1 illustrates the relation of O consumption to time in a 16 hour culture. The experiment shown in this figure was continued to 48 hours total time. The rate merely approached zero asymptotically.

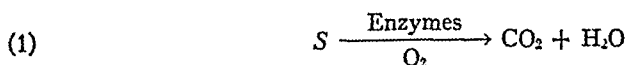
At the close of the experiment only a slight increase was detected in the number of cells taking up methylene blue and 0.05 cc seeded into fresh Williams' medium grew prolifically

The KOH in the inset of the Warburg vessel was renewed at intervals with no consequent change in rate, and the addition of filter paper to the well to present greater surface for absorption of the CO₂ produced (Dixon and Elliott, 1930) was without effect. The partial pressure of oxygen was maintained well above the critical value throughout the experiment (Tang, 1933, Tang and French, 1933, French, 1934) by frequently opening the vessels to the air, removing the side arm stoppers, and drawing air through (*cf* French, 1934). It is probably safe to assume the critical p_{O_2} for this endogenous reaction to be at least as low as that for the respiration of added sugar. (Stier (1935-36) shows that this assumption is probably justified.)

We feel that the decline in rate of respiration has real significance and is not due to the death of any significant percentage of the cells or to technical artifacts

The Order of the Reaction

The shapes of both the differential and integral curves (*cf* Figs 1 and 2) for the declining rate type of curve were regular enough to suggest kinetic analysis. We assume that a single reaction is involved, *viz* the decomposition of some substrate, designated for the present as *S* (*Cf* French, Kohn, and Tang, 1934-35, for an illustrative case where more than one reaction is involved). The total amount of O₂ consumed or of CO₂ produced was assumed a direct measure of the amount of internally stored substrate utilized. Since the R/Q (CO₂/O₂) equals 1 throughout the course of the reaction (*cf* Fig 2) the products of the reaction are designated provisionally as CO₂ and H₂O. We may then write the decomposition of *S* as



This predicts that the concentration of substrate might limit the rate of O₂ consumption and of CO₂ production and that the reaction is first order with respect to time as long as neither the partial pressure of O₂ nor the enzyme concentration is limiting. That the rate of autofermentation of living yeast is limited by the concentration of

substrate has been suggested many times (Harden and Paine, 1912, Harden, 1932) and most recently by Belitzer (1934) Our evidence verifies this suggestion

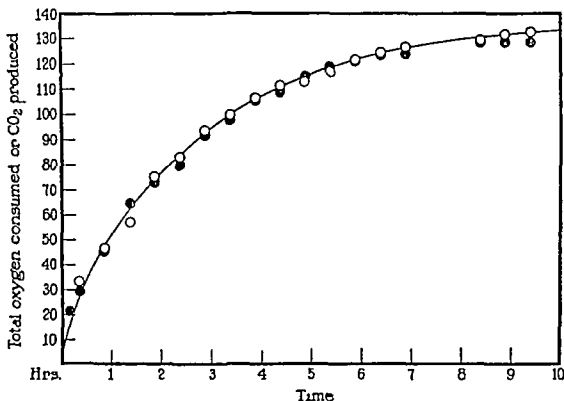


FIG 2 Integral curve showing the total amounts (c mm) of O₂ consumed and CO₂ produced as a function of time at 23°C in M/15 KH₂PO₄. The open circles represent O₂ consumption and the solid circles CO₂ production. The RQ = 1 throughout (Strain 4360 cultured at 25°C)

Data obtained in 15 experiments were tested by the following method

Let

y = c mm O consumed in a time, t

A = total c.mm O₂ consumed or CO produced \therefore the asymptotic value

a = original concentration of substrate S

x = amount of substrate consumed in t minutes

k = velocity constant for decomposition of S

k = velocity constant for oxygen consumption and CO₂ production

If the process is first order, \therefore pseudo unimolecular,

$$(2) \quad \frac{dx}{dt} = K(a - x)$$

which gives on integration the usual expression

$$(3) \quad k = \frac{1}{t} \ln \frac{a}{(a-x)}$$

A , the asymptotic value for the O_2 consumed or CO_2 produced, obtained from the integral curves, may be taken as proportional to the total amount of substrate S , and y , the amount of gas exchanged in the time t may be taken as proportional to x . In other words

Let

$$y = cx$$

$$A = ca$$

Then

$$(4) \quad \frac{dy}{dt} = \frac{k}{c} (A - y) = k'(A - y),$$

and

$$(5) \quad y = A(1 - e^{-k't}),$$

whence we obtain the expression

$$(6) \quad \ln \left(1 - \frac{y}{A} \right) = -k't$$

Plotting $\ln \left(1 - \frac{y}{A} \right)$ against time should then give a straight line with slope $-k'$. Fig. 3 is an example of the fit obtained when data for the endogenous respiration of young cells are plotted in this manner. The velocity constant $-k'$ was calculated as 2.303 times the slope of the line since logarithms to the base 10 were used. The data used in plotting Fig. 3 are shown in Table I. Log rate against time also gives a linear relation, but we preferred the method shown in Table I and Fig. 3 in order to obtain the first order velocity constant.

A further test for the first order character of such reactions has been suggested in another connection by French, Kohn, and Tang (1934-35) for *Chlorella pyrenoidosa*, viz plotting the rate at any time, dt , against the total amount of oxygen consumed in any total time, t . This method makes it unnecessary in the case of a single reaction to

determine the asymptotic value for y ($\pm c A$), and is useful when velocity constants are not desired as a quick test for the order of the reaction. It has further application when a constant basal rate obscures the order of a superimposed reaction.

The validity of the assumption that the concentration of substrate S is the rate limiting concentration when oxygen consumption or CO_2 production is measured has been subjected to further tests. It has been shown already that $p\text{O}_2$ or concentration of oxygen in the cell is probably not rate limiting. Following the data presented by Rubner (1913) and discussed by Rahn (1932)⁵, showing

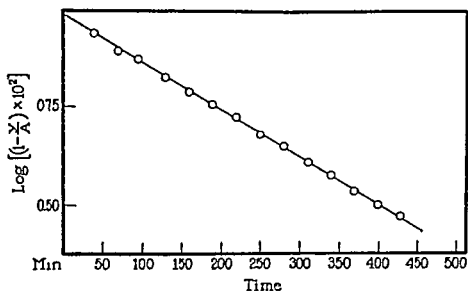


FIG. 3 Test for the first order character of the declining rate portion of the "dissimilation curve". The points plotted are those calculated in Table II. The value of the first order velocity constant $-k = 0.0029$ (Experiment at 20°C GM yeast used.)

that yeast suspended in non nitrogenous media continually loses organic nitrogen to the medium, it might be supposed that the decline in rate is a reflection of the gradual deterioration of the enzymes involved signifying a first order decomposition of the catalysts.

This possibility was tested by adding a few crystals of dextrose to the vessels at the end of a long experiment. Within 5 minutes the constant high rate of O_2 consumption and CO_2 production characteristic of yeast in the presence of a plentiful supply of dextrose was reached, and was found to agree quantitatively with that normally obtained with a fresh sample of yeast. A similar experiment is reported by Belitzer (1934). Unless we make the unwarranted assumption that the enzyme or enzymes concerned with endogenous respiration are both inde

pendent of and more easily decomposed than those involved in exogenous sugar metabolism, we must conclude that enzyme concentration does not limit the rate of endogenous respiration during the declining portion of the dissimilation curve. This constancy of the power to utilize exogenous sugar was shown by Buchner and Mitscherlich (1904) and by Iwanowski and Brzezinski (1934).

The velocity constant for endogenous respiration has a high temperature coefficient (*cf* later papers), thus eliminating possible physical limitations on the rate, *e.g.*, diffusion.

TABLE I

Sample Calculation for Determining the First Order Velocity Constant

Temperature = 20°C *		$A = 130 \pm 5$ c mm oxygen (or CO ₂)		
Time	y	$\frac{y}{A}$	$1 - \frac{y}{A}$	$\text{Log} \left[\left(1 - \frac{y}{A} \right) \times 10^2 \right]$
<i>min</i>	<i>c mm</i>			
40	15.8	0.122	0.878	1.9435
70	27.0	0.208	0.792	1.8987
95	34.0	0.262	0.738	1.8681
130	43.0	0.331	0.669	1.8254
160	50.8	0.391	0.609	1.7846
190	55.5	0.427	0.573	1.7582
220	63.5	0.488	0.512	1.7093
250	67.5	0.519	0.481	1.6822
280	71.7	0.552	0.448	1.6513
310	76.5	0.588	0.412	1.6149
340	80.8	0.622	0.378	1.5775
370	84.5	0.650	0.350	1.5441
400	88.0	0.677	0.323	1.5092
430	90.8	0.698	0.302	1.4800

* Most of the experiments were performed at 25°C, but the shape of the curve is more clearly discernible when plotted from data obtained at the lower temperature.

† Characteristic asymptotic value for fresh GM yeast.

The asymptotic value, A , varied considerably from culture to culture as well as with the strain of yeast used. It was always higher for the 4360 strain which had just been removed from its culture medium than for the GM yeast. In the case of the latter there is good evidence that some of its carbohydrate reserves were utilized during transit and storage. The characteristic values of A were 400–200 c mm O₂ for Strain 4360 and 225–75 c mm O₂ for GM yeast. If the cultured cells of Strain 4360 were not washed completely an initial

fraction of the O_2 consumed is, of course, referable to the utilization of the last traces of hexose sugar. This is easily detected as a high initial rate of respiration followed by a sudden drop and a sharp bend on the first order plots. The amount of this fraction must be subtracted from

TABLE II
Endogenous Respiration Showing the Presence of 'Residual' Hexose

Time	Rate O_2 uptake	Time	Rate O_2 uptake
m n	c mm / 10 min	m n	c mm / 10 m n
25	35.7	125	7.5
45	36.8	145	5.3
65	16.8	185	5.5
85	9.2	225	5.1
110	8.9	265	4.7
		305	3.7
		345	3.1

GM yeast in M/15 KH_2PO_4 (pH 4.5), experiment at 25 C

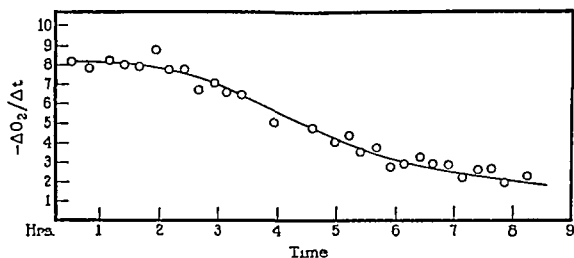


FIG. 4. The relation of the rate of O_2 consumption to time for a 4-day culture. Note that the initial rate is lower than the initial rate for younger cultures (*cf* Fig. 1), and remains constant for almost 2 hours. The succeeding period of declining rate is only approximately first order. (Experiment at 25 C, M/15 KH_2PO_4 , Strain 4360 cultured at 25 C.)

the total amount of O in determining A . Such a case is illustrated in Table II.

Older Cultures

The relation of the rate of dissimilation to time in a non-nutrient medium seen in either strain of yeast grown for more than *ca.* 24 hours

in Williams' medium at 25–26°C differs from that seen in younger cultures. The initial rate of O_2 consumption and CO_2 production is lower, and remains constant for a period of time varying with the age of the culture, finally falling off slowly towards zero (*cf* legend, Fig 5). Figs 4 and 5 are differential curves for the time-rate relation in 4 and 10 day old cultures respectively. It will be noted that the value of the y intercept decreases with the age of the culture. A gradual fall in the initial rate of O_2 consumption when yeast metabolizes added dextrose was also observed as a function of age of the culture. These phenomena will be discussed in a separate account.

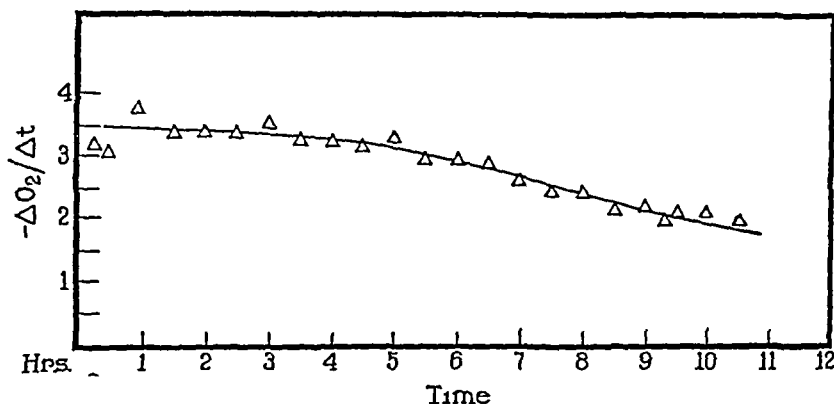


FIG 5 The relation of the rate of O_2 consumption to time for a 10 day culture. Comparison with Figs 1 and 4 will show that the initial rate is lower than in either of the previous cases and remains constant for a longer time (4–5 hours). Only a part of the declining portion of the rate curve is shown. It is characteristic that this portion of the curve often becomes less like a first order decline as the culture age increases. This awaits further investigation. See text (Experiment at 25°C, $M/15$ KH_2PO_4 , Strain 4360 cultured at 25°C).

The R/Q is equal to 1 for the older cultures as well as for the younger cultures, and the characteristics of the endogenous metabolism (considered in the following paper) indicate that the two portions of the dissimilation curve are definitely aspects of the operation of a single system. The instantaneous rate of respiration in one phase is controlled by the substrate concentration and in the other presumably by the enzyme concentration. In this connection attention is called to the recent work of Hopkins and Roberts (1935) on the kinetics of alcoholic fermentation in the presence of glucose. At low sugar concentrations and high yeast concentrations a first order relation was

found between the rate of fermentation and time. They show that the various phases seen in the kinetics of alcoholic fermentation are

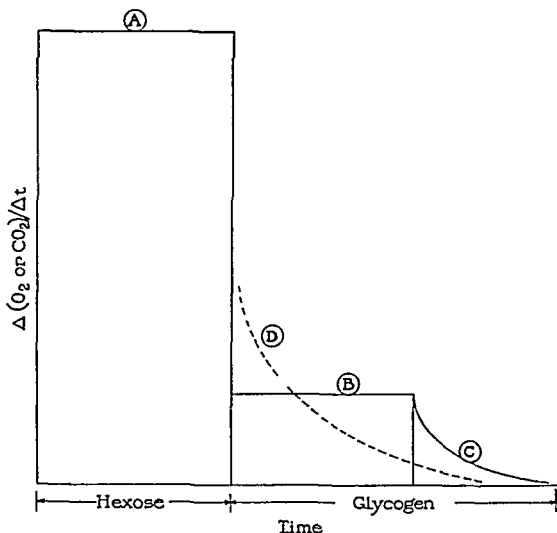


FIG. 6 Diagrammatic summary of the types of respiration seen in bakers' yeast. Part A represents the relation seen when bakers' yeast is suspended in dextrose buffer solution or when the yeast, suspended in pure phosphate buffer, dissimilates the last traces of dextrose remaining within the cell (or adsorbed on its surface cf Wertheimer 1934). In the former case the high rate of respiration is maintained for several hours; in the latter the rate falls rapidly to the lower levels shown. Usually the rate of CO_2 production for part A is higher than the rate of O_2 consumption, i.e., the RQ is greater than 1. Parts B and C refer to the dissimilation curve as seen for cultures more than 24 hours old—a period of constant rate followed by a declining rate. Part D refers to the first order decline characteristic of young cells in phosphate buffer. The $RQ = 1$ for parts B, C and D.

but special cases of a general kinetic relationship between the concentration of added glucose and "enzyme" (yeast concentration) ...

A diagrammatic summary of the types of respiration seen in bakers' yeast is given in Fig 6

It was almost always possible by proper choice of asymptote to obtain first order constants and satisfactory linear relations, except in a few isolated cases (4 out of 19 experiments) where the rate suddenly assumed a constant low value for several hours instead of approaching zero. The RQ remained ≈ 1 throughout the entire experiment in two of these cases, the temperature coefficient was low, indicating a peculiar phase in the mobilization of stored substances in which some physical process, possibly diffusion of the substrate from the storage "depots" to the active enzyme surfaces, became rate-limiting. These cases were both obtained with young cultures having higher stores than normal. However,

TABLE III
Change of Respiratory Quotient with Time

Time	$-\Delta O_2/\Delta t$	$\Delta CO_2/\Delta t$	RQ
<i>min</i>	<i>c mm O₂/10 min</i>	<i>c mm CO₂/10 min</i>	
20	2.5	2.6	1.04
45	2.1	2.0	0.95
215	0.9	0.86	0.95
325	0.95	0.85	0.90
385	0.56	0.51	0.91
470	0.58	—	—
1070	0.61	0.44	0.72
1170	0.57	0.42	0.74

The rates of respiration are averages from two vessels (average deviation as per cent of the mean $= \pm 3$, maximum deviation $= 6$ per cent). From integral curves of these data, $RQ = 0.89$ to 0.90 between 470 and 1170 minutes (Experiment at $25^\circ C$, $M/15$ KH_2PO_4 , GM yeast).

other young cultures with high concentrations of stored material did not behave in this way.

In two other experiments the RQ gradually lowered to values as low as 0.72 and the rates of respiration were not fitted by a first order equation (cf Table III). This was seen when cells having very low concentrations of stored material were employed, e.g. GM yeast stored 2 days at $20^\circ C$ or several days at $7^\circ C$, it is possibly associated with the utilization of non-carbohydrate materials (cf Meyerhof, 1925, and Geiger-Huber, 1934).

IV

The analysis presented above indicates that the rate of endogenous respiration is sooner or later limited by the concentration of some

substrate which we designated as *S*. Evidence from the literature, already given, identifies substance *S* as a carbohydrate, probably glycogen. Additional evidence is afforded by the following statements.

Careful chemical analyses of GM yeast performed at the Fleischmann Research Laboratories by Dr Charles N. Frey (private communication) gave the following results:

1 The amount of CO₂ produced in the absence of added sugar was roughly proportional to the glycogen content of the cells.

2 The relative per cent total nitrogen, in milligrams per gram dry weight, *increases* with time in the absence of added sugar, becoming constant when the glycogen had completely disappeared. This rise corresponded *quantitatively* with the value expected if the stored carbohydrate were the only significant substrate disappearing during the experimental period.

Since analyses for the glycogen content of yeast are laborious and apt to be uncertain, this becomes independent evidence of a corroborative sort that the main substrate during the dissimilation of living yeast cells is not protein or other nitrogenous material. Through the kindness of Dr P. R. Gast of the Harvard Forest we obtained some preliminary data for the total nitrogen content of our No. 4360 strain of yeast before and after dissimilation. Our results agree with those obtained by Frey for GM yeast.

We have also made microscopic examinations of the glycogen content of yeast before and after dissimilation, using the iodine staining technique employed by Harden and Rowland (1901) and by Wager and Peniston (1910). A progressive decrease in the amount of red brown staining material was observed during the course of the dissimilation curve (*cf.* Wager and Peniston, 1910, for further details). However, more direct evidence is necessary before it is decided unconditionally that the substrate concerned is uniformly glycogen and no other carbohydrate.

A reexamination of the enzyme systems involved in the endogenous metabolism of bakers' yeast is presented in the following paper.

SUMMARY

The process of endogenous respiration of two strains of bakers' yeast, *Saccharomyces cerevisiae*, was examined kinetically. The rate of respiration with respect to time in a non-nutrient medium was found to exhibit two phases: (a) a period of constant rate of O₂ consumption and CO₂ production ($R/Q = 1$) characteristic of cells with ample con-

centrations of stored material, (b) a first order decline in rate of respiration with respect to time, where the rate was proportional to the concentration of some substrate, S ($R/Q = 1$ throughout second phase) The nature of this substrate was reexamined and the evidence summarized confirms the notion that it is a carbohydrate, probably glycogen These phases of endogenous respiration were shown to depend upon the age of the culture and the amount of substrate available

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THE METABOLIC SYSTEMS INVOLVED IN DISSIMILATION OF CARBOHYDRATE RESERVES IN BAKERS' YEAST

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I

In a previous paper concerned with a kinetic analysis of endogenous respiration of bakers' yeast (Stier and Stannard, 1935-36) it was shown that the rate of respiration was a function of the time in the "non nutrient" medium, the age of the cells, and their previous history. The rates of both O_2 consumption and CO_2 production were equal under almost all conditions. Evidence was summarized to show that the dissimilated substrate is a carbohydrate, probably glycogen. The statement was made that the term *endogenous respiration* is preferable to "autofermentation" in referring to the metabolic utilization of stored glycogen in intact bakers' yeast cells. This change in terminology was based mainly upon the results of investigations reported in the present paper.

It is usually assumed that the decomposition of stored glycogen proceeds within the yeast cell by the same mechanisms as the respiration and fermentation of simple carbohydrates placed in the external medium¹. We will show in this account that dissimilation of the available carbohydrate reserves proceeds by way of a "unit" chain of reactions resulting in a complete oxidation of the carbohydrate to CO and H_2O . Since "pure" respiration only is involved we may speak of "the respiratory character of endogenous metabolism" or may refer directly to the process as "endogenous respiration". We will also show that the endogenous chain of reactions behaves as a distinct functional unit only when the structural integrity of the cell is maintained. Its unitary character is thus the property of a "chambered architecture" of the yeast cell (*cf* Hofmeister, 1901).

¹ By fermentation we mean anaerobic CO_2 production and the accumulation of alcohol in the medium.

II

The Effect of Specific Inhibitors

The Effect of KCN—On the basis of evidence from the literature of this subject it is highly probable that the O_2 consumed during the endogenous breakdown of glycogen is transported by means of the intracellular hematin compounds of yeast whose activity is thought to be specifically inhibited by the presence of cyanide (also by CO , H_2S , NO) For summaries dealing with cyanide inhibition of the respiration of bakers' yeast suspended in sugar-free media *cf* Warburg (1927) and Keilin (1929) Keilin (1929) has shown that the "activated" substrate molecules obtained from the internal stores of yeast reduce the cytochrome system Recently Erwin Haas (Warburg, 1934) has obtained data supporting the view that the pheohemincytochrome system is the *chief* oxygen-transporting system of the bakers' yeast cell

We have reinvestigated the effect of KCN on endogenous respiration especially examining its effect on the endogenous production of CO_2

The yeast was suspended in $M/15$ KH_2PO_4 solution at pH 4.5 and adjusted to standard density by means of the photoelectric densitometer (*cf* Stier, Arnold, and Stannard, 1933-34) 2 ml of this suspension (approximately 10 mg dry wt) were added to each of several Warburg respirometer vessels arranged in pairs One pair of vessels was used for each poison concentration After thermal adaptation a 30 to 60 minute period was allowed during which the rates of respiration of all the sets of vessels were determined simultaneously The latitude of "experimental variation" could be thus ascertained for the given set of conditions KCN was then added through the side-arm of the vessel by means of a 1 ml hypodermic syringe graduated in hundredths of a milliliter The volume of KCN added was 0.5 ml, making the total volume of fluid in the vessel 2.5 ml The concentration added was therefore five times that desired in the experiment

After 90 to 100 minutes, a measured amount of dextrose crystals was added (calculated to be *ca* five times the quantity required to saturate the enzyme surfaces, *cf* Harden, 1932, Geiger-Huber, 1934), and the measurements were continued another 90 to 120 minutes Usually, at the lower concentrations of poison, due to the large number of yeast cells used for the measurement of endogenous respiration, the rate after sugar had been added was too high for accurate measurement of the respiratory rate For the determination of these rates parallel vessels containing a lower concentration of yeast suspended in 2 per cent dextrose-buffer solution were employed Values of Q_0 and Q_{CO_2} were calculated by using factors

It will be noted in Table I that, in agreement with Warburg (1927), the per cent inhibition of endogenous respiration is lower at equivalent cyanide concentrations than the per cent inhibition of the O_2 consumption in a sugar-containing medium. Warburg's suggestion that this is due to "unsaturation" of the enzyme surfaces with substrate in the former case is supported by data presented below. Obviously as the dissimilation curve progresses and the concentration of internal

TABLE II
Inhibition of Endogenous O_2 Consumption by 2×10^{-3} Molar KCN As a Function of Time, at 25°C

	Time	Control vessel — $\Delta O_2/\Delta t^*$	KCN vessel — $\Delta O_2/\Delta t^*$	Inhibition
	<i>min</i>			<i>per cent</i>
Zero order portion†	55	—	KCN added	—
	75	21.0	1.0	95
	85	20.5	1.4	93
	95	20.5	1.2	94
	105	14.2	1.2	91
First order portion†	115	11.7	1.2	89
	125	9.8	1.5	85
	135	9.3	1.5	84
	145	7.1	1.5	79
	155	Sugar added	Sugar added	—
	165	58.1	1.2	98

A 43 hour culture of Strain 4360

* C mm O_2 /10 min

† Portions of dissimilation curve, *cf* Stier and Stannard, 1935-36, Fig. 6. The endogenous CO_2 production followed exactly the O_2 consumption under all experimental conditions imposed during both the constant rate and declining rate portions of the dissimilation curve. This is significant with regard to the unitary behavior of the endogenous chain of reactions (*cf* Section V).

substrate diminishes, the per cent inhibition should decrease with time, since the degree of unsaturation is increasing. That this occurs is shown in Table II.

An affinity constant, k , can be calculated, representing the ratio of the respective affinities of the poison and oxygen for the KCN-labile enzyme surfaces (Warburg, 1927). In cases where these enzyme surfaces are evidently not completely saturated with substrate a cor-

rection for the unsaturation should be applied (*cf* Warburg, 1927, for CO inhibition, and Runnstrom, 1928 and 1934, for the inhibition of respiration of sea urchin eggs by KCN), since considerable enzyme might enter into combination with the cyanide before any effect on the rate can be measured. For these cases a "saturation factor," ϵ , is introduced and the affinity constant, k' , obtained. We have made these calculations for endogenous respiration, the value of k' was *ca* 10^4 for both the endogenous and the exogenous respiration, this value is usually associated with oxygen transporting systems containing the intracellular hematin compounds (*cf* Runnstrom, 1928). Lower values of k' were obtained in the case of yeast cultured for more than 2 days at 25°C in Williams' medium. The data pertaining to these older cultures will be presented in another place.

In this connection Emerson (1926-27) reports that the respiration of *Chlorella* in a glucose free medium is not inhibited by 10^{-4} N HCN, while the respiration in the presence of glucose is *ca* 60 per cent inhibited by the same concentration of the poison. A superficial similarity to the case in yeast is obvious. There are not sufficient data available to determine whether a separate O_2 transporting system exists for the two types of respiration or whether the saturation factor operative in the case of yeast plays a part here as well.

The existing data, therefore, leave little doubt that the same oxygen transporting system is involved in both endogenous and exogenous respiration. Furthermore, they indicate that the endogenous chain of reactions does not produce CO by any other method than that afforded by a respiratory type of system. Unlike exogenous metabolism, it cannot produce CO in the presence of cyanide of sufficient concentration to inhibit its oxygen consumption. The implication is that "fermentation" is *not* involved in the dissimilation of carbohydrate reserves in living yeast cells.

The Effect of Monoiodoacetic Acid and Related Compounds

The specific effect of low concentrations of monoiodoacetate and other substances of this type on alcoholic fermentation was demonstrated by Lundsgaard (1930). In concentrations of 10^{-4} molar monoiodoacetate completely inhibits fermentation in dried yeast, leaving respiration unhampered. The R/Q approaches 1, and we

have then "pure respiration" However, secondary effects soon lower the R/Q still further With higher concentrations of the poison the oxygen consumption (of living yeast) is also affected (Nilsson, Zeile, and von Euler, 1931)

The effect of monoiodoacetic acid on endogenous respiration was studied by the same technique as that discussed above for KCN

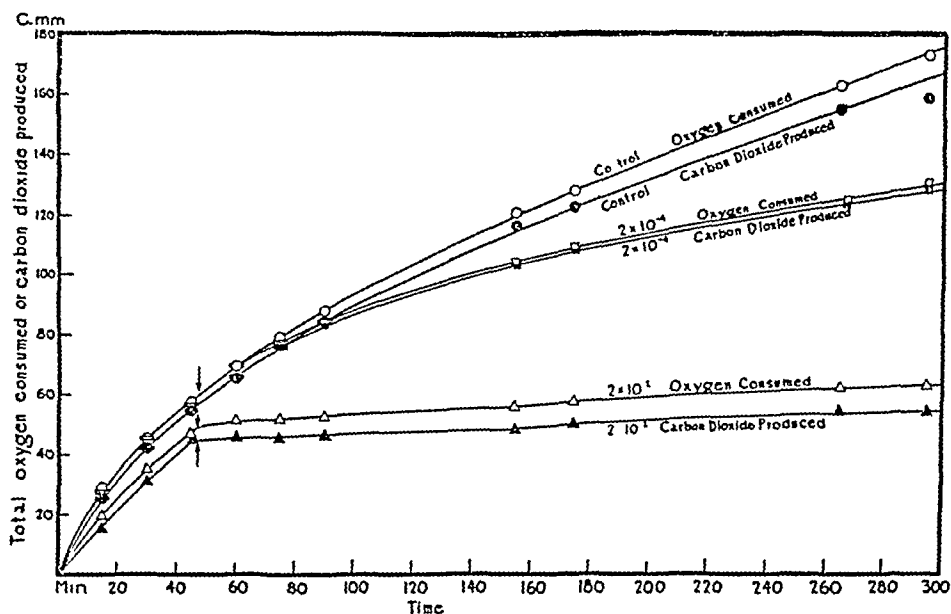


FIG 1 The effect of monoiodoacetic acid in two concentrations (2×10^{-4} molar and 2×10^{-2} molar) on the endogenous respiration of yeast The open symbols represent oxygen consumption and the solid symbols CO_2 production (in cmm) The poison was added at the point marked with arrows Note that the curves for oxygen consumption and CO_2 production have identical slopes Note also the lag in the effect of 2×10^{-4} molar iodoacetic acid The yeast was suspended in pure phosphate buffer at pH 4.5 Intermediate concentrations of the poison have intermediate effects (Strain 4360, cultured for 43 hours at $25^\circ C$, Experiment at $25^\circ C$)

The initial and final pH of the medium was 4.5 The effects on exogenous metabolism were also studied in order to compare the action of these substances on both systems and if possible discover the interrelationships existing between the endogenous and exogenous systems

In Fig 1 it is seen that endogenous O_2 consumption and CO_2 pro-

duction are both affected, the R Q remaining 1 throughout the entire experiment. The effect is thus the same as that obtained with KCN. Note also that there is a "lag" in the action of the lower concentration of the poison. Thus, with a concentration of 2×10^{-4} molar 40 minutes elapsed before any appreciable decline in respiration occurred. The inhibition of both O_2 uptake and CO_2 production was only 60 per cent at the end of that time. A 2×10^{-3} molar solution inhibited the endogenous respiration immediately and almost completely.

These findings are in sharp contrast to those obtained when substances containing the iodoacetyl group are added to yeast in the presence of dextrose. It has already been shown that the fermentation process (*i.e.*, alcohol production and anaerobic CO_2 production) is quickly inhibited at concentrations as low as 10^{-4} molar (*cf.* Lunds gaard, 1930, Nilson, Zeile, and von Euler, 1931). We have repeated these experiments on our strains of yeast and have found that a concentration of 2×10^{-4} molar was sufficient in general to induce immediately a 98 per cent inhibition in the over all rate of CO_2 production. The O_2 consumption remained unaffected for 45 minutes after the rate of CO_2 production had become negligible. At the end of this period, however, the rate of O_2 consumption suddenly fell off to 10 per cent of its original value. The per cent inhibitors were then 98 and 90 for CO_2 and O respectively. These results are illustrated in Fig. 2.

The nature of the lag period is uncertain. It is apparent that it decreases as the concentration of monoiodoacetic acid increases and so might be a "penetration phenomenon." Goddard (personal communication) has prepared monoiodoacetamide as a neutral molecule bearing the iodoacetyl group but more "lipoid soluble" and hence more rapidly penetrating than either monoiodoacetic acid or acetate. Furthermore, Goddard (1935-36) has given evidence that this compound penetrated the ascospores of *Neurospora tetrasperma* more rapidly than the iodoacetate. Kohn (1935-36) compared the action of monoiodoacetic acid, acetate, and monoiodoacetamide on photosynthesis in *Chlorella pyrenoidosa* and found that the amide penetrated most readily. Dr. Goddard kindly gave us a sample of the amide, preliminary investigations indicate that the lag

in the effect of the iodoacetyl group on the endogenous respiration of yeast is greatly reduced by the use of this compound

However, even after the "penetration period" has apparently been completed the rate of endogenous production of CO_2 is not inhibited to the same extent as the exogenous CO_2 production. This is illustrated in Table III. (Also compare the per cent inhibitions at equiv-

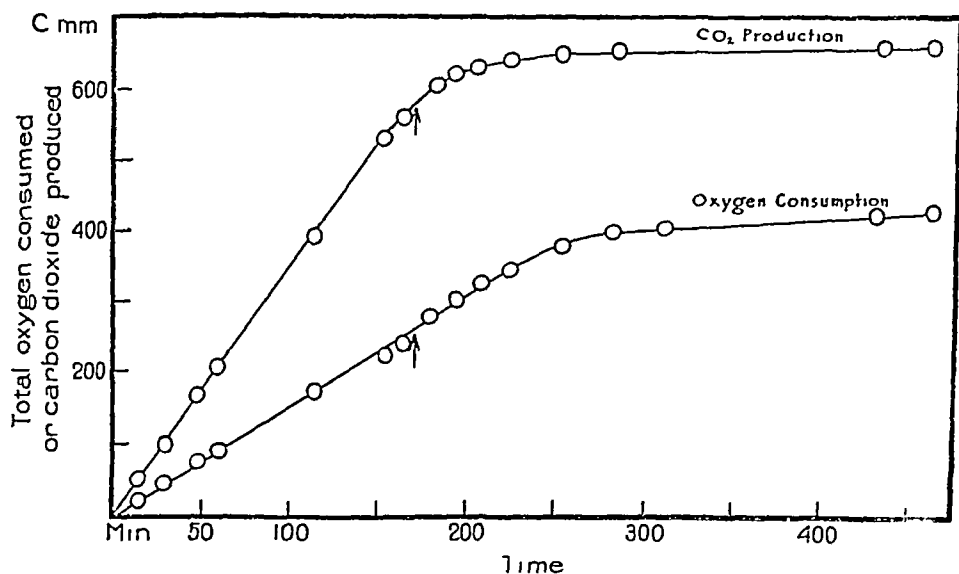


FIG 2 The effect of 2×10^{-4} molar monoiodoacetic acid on the oxygen consumption and CO_2 production of yeast suspended in dextrose-buffer solution. The poison was added at the point marked with arrows. Note the higher slope of the carbon dioxide curve indicating a higher rate of CO_2 production than of oxygen consumption, and thus an RQ greater than 1. The inhibition sets in much more rapidly in the case of CO_2 production. The time for this lag in the effect on O_2 consumption is identical with the time for penetration to the endogenous system (cf Fig 1). The rates of the controls (not shown) remained constant (Strain 4360, 25°C).

alent poison concentrations in Figs 1 and 2). This effect, as well as the lag period, may be a reflection of the relative inaccessibility of the endogenous chain of reactions, or, the saturation factor discussed in connection with the inhibition by KCN may be operative as an additional complication. The action of the iodoacetyl group is not specific enough to warrant calculations such as those discussed above.

for the action of KCN (*cf* Michaelis and Schubert, 1934, Runnström and Michaelis, 1934-35)

We believe that the data just presented support the view that endogenous respiration of bakers' yeast is a pure respiration. Apparently the alcohol producing portion of the zymase complex does not function in the absence of sugar (dextrose) in the external medium.

TABLE III

Effect of Moniodoacetic Acid on Endogenous and Exogenous CO₂ Production (Aerobic) at the End of the Penetration Period

	Endogenous CO ₂ production			Exogenous CO ₂ production	
	QCO		Inhibition	QCO ₂	Inhibition
			per cent		per cent
Nov 9	Control	2.66	—	34.5	—
(3 day culture)	2×10^{-4} mol	1.03	61.3	0.6	98.2
Nov 12	Control	2.73	—	—	—
(3 day culture)	2×10^{-4} mol	1.05	61.4	—	—
	2×10^{-3}	0.33	88.0	—	—
Nov 23	Control	0.45	—	43.4	—
(14 day culture)	2×10^{-4} mol	0.17	63.0	5.1	88

QCO₂ = c mm CO₂ produced per mg dry yeast per hour. Experiment at 25 C. Strain 4360

III

Anaerobic CO₂ Production

As a further test of the purely respiratory character of the endogenous chain of reactions the anaerobic CO₂ production was measured in Warburg respirometers.

The ordinary commercial nitrogen was passed through a reduction valve into Fieser's solution (Fieser, 1924) to remove the last traces of oxygen since even very low partial pressures of oxygen interfere with the determination of anaerobic CO₂ production. The small amount of H₂S formed was removed from the gas stream by connecting an absorption tower of saturated lead acetate solution (Tang 1932-33) in the chain. The gas was then saturated with water vapor and passed through the Warburg vessel and its manometer for 7 to 8 minutes with vigorous shaking. This has been shown to be sufficient time for complete equilibration (Krebs 1928). Then, with a positive pressure remaining in the apparatus the stop-cocks were closed stoppers inserted and the manometer sets transferred

to the thermostat for thermal adaptation. Controls were run for oxygen consumption and aerobic CO_2 production. As a preliminary test of the method the Q_{O_2} , Q_{CO} in air, and Q_{CO_2} in nitrogen were determined in a 2 per cent dextrose-phosphate solution and the values checked with those appearing in the literature for fermentation by bakers' yeast.

TABLE IV

O₂ Consumed and CO₂ Produced as a Function of Time under Aerobic and Anaerobic Conditions, at 25°C

Time	Total* O ₂ consumed	Total* CO ₂ produced (aerobic)	Total* CO ₂ produced (anaerobic)	Time	$\frac{\text{c mm CO}_2^\dagger}{10 \text{ min}}$ (anaerobic)
	<i>c mm</i>	<i>c mm</i>	<i>c mm</i>	<i>min</i>	
11 20	0 0	0 0	0 0	0	0
11 30	7 1	7 5	0 3	10	+0 28
11 40	15 5	16 0	0 5	20	-1 10
11 50	22 4	22 3	0 8	40	0 00
12 00	28 5	28 7	1 7	70	0 00
12 15	36 7	36 8	1 7	95	0 00
12 30	43 8	43 4	1 7	150	+0 85
12 45	49 9	49 0	2 8	175	+1 40
1 00	55 1	55 8	3 7	185	-0 57
—	—	—	—	201	+0 57
—	—	—	—	215	-0 90
2 00	70 4	70 5	5 1	230	+0 90
—	—	—	—	245	0 00
—	—	—	—	260	+0 60
3 15	79 9	80 0	8 8		
Totals in 235 min	79 9	80 0	8 8		

* Experiment Nov 27 on a 3 day culture of Strain 4360

† Experiment Dec 15 on a 12 day culture of Strain 4360. Simultaneous measurements of the aerobic O_2 consumption and CO_2 production indicated that an ample supply of stored material for respiratory utilization was present.

The CO_2 production under anaerobic conditions was found to be practically negligible. If the last traces of hexose were incompletely washed away from the cells, CO_2 would be produced anaerobically in $\text{M}/15 \text{ KH}_2\text{PO}_4$ solution for about 1 hour and then would suddenly cease. The rate of CO_2 production was, however, about 0.5 that of the aerobic rate. In data given by Geiger-Huber (1934) one sees a similar reduction, the rate of anaerobic CO_2 production was about 0.1

of the rate of aerobic CO_2 production for yeast suspended in $\text{M}/15$ KH_2PO_4 . We attribute this small initial production of CO_2 to the utilization of the last traces of hexose or of some intermediate substrate (prepared by the exogenous system while the yeast was still in the sugar containing medium) which the fermentation mechanism is able to utilize. The data presented in Table IV (Experiment November 27) are from the most complete experiment performed. In Experiment December 15 (Table IV) no residual hexose was involved since the culture was 12 days old. Reference to Table VI should also be made.

Other instances showing a lack of anaerobic CO_2 production of cells under starvation conditions can be found in the literature. Meyerhof (1912a and b) showed that if respiring blood cells and aerobic bacteria are deprived of O_2 there is no measurable CO_2 production and no perceptible heat production. Lyon (1923-24) noted that *Elodea canadensis* did not produce CO_2 under anaerobic conditions. Unpublished observations of P S Tang (*cf* French, 1934) indicate that the green alga, *Chlorella pyrenoidosa* will not decompose its photosynthetically stored carbohydrates in the absence of oxygen. Tang subjected *Chlorella* in Warburg respirometers to an atmosphere of nitrogen. No deflection of the manometers was observed during an 18 hour period. This phenomenon needs further consideration, especially since it has an important bearing on the real nature of the 'energy for maintenance' assumed to be a necessary condition of the living state.

That the lack of "anaerobiosis" in endogenous metabolism is a real phenomenon and not a technical artifact was tested in several ways.

- 1 The cells were not 'dying' so far as could be determined by staining them with methylene blue after several hours in an atmosphere of nitrogen. A small portion seeded into Williams' medium produced the same 'crop' as the control.

- 2 On the admission of oxygen rapid CO_2 production immediately began with a rate equal to or even 50 per cent higher than the initial rate of the control (aerobic). The same results were obtained for oxygen consumption.

- 3 If the vessel was opened a few crystals of dextrose added, and then re-equilibrated with nitrogen $Q_{\text{CO}_2} = 250 \pm$, the normal value for anaerobic CO_2 production in bakers' yeast (Meyerhof 1925), was obtained.

- 4 The medium, $\text{M}/15$ KH_2PO_4 solution, was found to maintain its pH of 4.5 ± 0.05 during even 6 to 8 hours under anaerobic conditions.

- 5 2 per cent HCl placed in the side arm and tipped into the medium at the end of an experiment showed no sudden evolution of gas.

- 6 The two strains of yeast used (GM and No 4360, *cf* Stier and Stannard, 1935-36) behaved similarly.

V

We conclude that a purely respiratory system takes part in the dissimilation of carbohydrate reserves in bakers' yeast. The striking feature is the relative simplicity of organization which it exhibits provided the complex structure of the cell is kept intact. From the evidence presented it seems reasonable that, unlike the situation in the metabolism of added sugar, there is one "unbranched" chain of reactions to which the alcohol-producing portion of the zymase complex is not functionally coupled. The oxygen-transporting system in endogenous respiration is the same as that engaged in exogenous metabolism.

On the basis of a kinetic analysis of endogenous respiration (*cf* Stier and Stannard, 1935-36) it was also concluded that the endogenous system behaved functionally as a unitary system of reactions. During the declining rate portion of the dissimilation curve the *instantaneous* rate of respiration is apparently a function of the concentration of the reacting materials. The rate constant for this portion is then the appropriate constant describing the activity of the unitary system of reactions. As a working hypothesis it is assumed that a serial alignment of reactions exists within the system—certainly a "functional alignment" of this type might be reasonably assumed. Furthermore, if we follow the hypothesis that some one process in a chain of reactions controls the activity of the chain (an outgrowth of Blackman's original suggestion (Blackman, 1905)), then the descriptive constant for the rate of decomposition of a relatively large amount of substrate can be used to reflect the physical and chemical *characteristics* of this pace-setting locus—even though we do not know its real nature or its actual position in the chain.

We are also suggesting that some locus in the endogenous chain of reactions limits the rate of respiration during the constant rate portion of the dissimilation curve. The instantaneous rate of respiration is obviously not limited by the substrate concentration, and probably not by the enzyme concentration, unless it can be shown that it does not remain constant throughout both phases of dissimilation. The experiments just reported show that the same unitary chain of reactions functions during both phases of dissimilation. Integrated action of the many simultaneous reactions operating during the constant

rate portion could be accounted for by assuming a rate-controlling step in the chain of endogenous reactions. In a succeeding paper we will show that the *same* locus is probably in control during both phases of dissimilation.

The conclusions given thus far were based almost entirely upon evidence obtained at a few temperatures in the limited range (20 to 25°C). Obviously, in any chain of reactions there is always the possibility that different steps may become rate limiting loci at different temperatures. This possibility was therefore investigated in order to make a general statement regarding the dynamical organization of the reactions involved in endogenous respiration, and the results form the subject of a succeeding paper.

SUMMARY

Evidence is presented showing that the dissimilation of carbohydrate reserves in two strains of bakers' yeast, *Saccharomyces cerevisiae*, is a purely respiratory process.

Endogenous respiration is KCN labile. Our own experiments together with various accounts and data given in the literature show that the same "oxygen transporting mechanism" functions in both endogenous and exogenous metabolism. However, the lack of sensitivity of the endogenous system of reactions to *low* concentrations of moniodoacetic acid, the absence of anaerobic CO₂ production, and the absence of alcohol production, demonstrate that fermentation is *not* involved in the dissimilation of the carbohydrate reserves.

Throughout the experiments the endogenous respiration behaved functionally as a unitary system of reactions. The O₂ consumption and CO₂ production were parallel at all times, i.e., the R/Q was consistently 1. Moniodoacetic acid and KCN in concentrations from 10⁻³ to 10⁻¹ molar affected both O₂ uptake and CO₂ production to the same extent. The only agents known to alter the value of the R/Q were those which disrupted the normal protoplasmic structure, viz: grinding the cells with sand, plasmolyzing them with toluol and hypertonic salt solutions or pressing them in a hydraulic press. These agents brought about a vigorous anaerobic CO₂ production accompanied by an accumulation of alcohol in the medium.

The unitary character of endogenous respiration is exhibited only

when the normal structure of the cell is kept intact, apparently it depends upon the maintenance of a chambered (or compartmental) architecture of the cell

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THRESHOLD INTENSITY OF ILLUMINATION AND FLICKER FREQUENCY FOR THE EYE OF THE SUN FISH

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I

Light which is interrupted with sufficiently high frequency appears continuous to the human eye. If at a given illumination the frequency of flashes is gradually decreased, the light impressions begin to be perceived separately at a certain flicker frequency. We therefore have the possibility of examining the relation between critical flicker frequency and illumination.

A large body of work has been done to determine critical flicker frequencies in relation to illumination for the human eye. In a recent series of papers (Hecht, Schlaer, and Verrijp, 1933-34) a complete account of the facts has been given. With organisms other than man, critical flicker frequencies have been determined for the eye of the dragon fly larvae (Salzle, 1932) and of the bee (Wolf, 1933-34). In both cases a reflex motion was used for the determination of threshold reaction at critical illumination. Since the method applied to the study of these two organisms is intrinsically different from the one used for the study of the human eye, an effort was made to use a similar reflex method for the study of an eye which is anatomically more similar to the human eye than that of the arthropods mentioned. For test object the sun fish *Lepomis* was chosen.

II

If a *Lepomis* is placed in a glass tank surrounded by a system of alternating opaque and translucent stripes it will react with a movement of its body to a displacement of the stripe system. The fish's reaction consists in a motion in the direction of the stripe movement.

Lyon (1904) proved that the response of the fish is a reaction to seen movement Grundfest (1931-32*a, b*) made use of this reaction in fishes for the study of sensitivity to spectral lights He found that *Lepomis* was particularly definite in its response Since a good deal of information was secured by Grundfest's experiments, *Lepomis* was used for the study of critical flicker frequencies

For observation a fish is placed in a cylindrical glass jar which stands on a glass-topped table (Fig 1) The jar containing the fish is surrounded by a glass cylinder on which black opaque paper stripes are pasted, leaving translucent spaces of equal width between them The striped screen is mounted on an axle which runs in brass bearings and can be driven by a motor at various speeds To make a

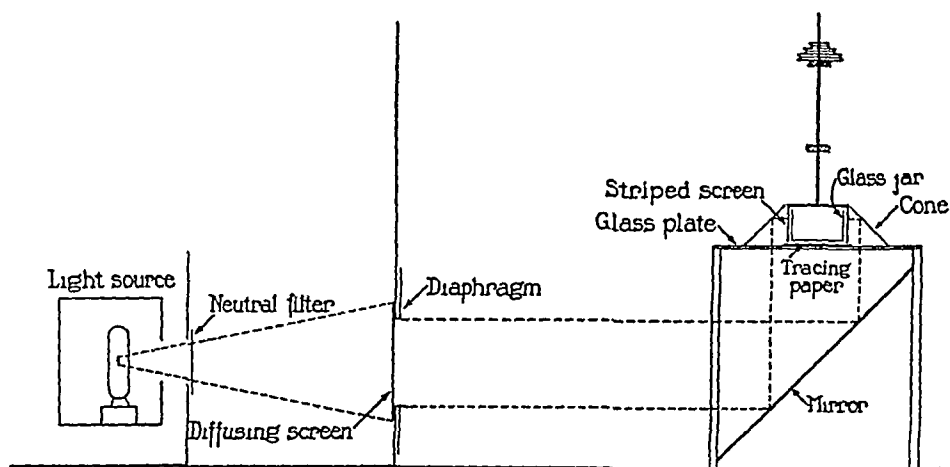


FIG 1 Diagram of apparatus for measuring threshold intensity of illumination at different flicker frequencies for the eye of the sun-fish

certain number of stripes pass in front of the fish's eye to produce a constant flicker frequency, two methods are employed First, the velocity of rotation of the screen can be varied by adjusting a rheostat controlling the voltage of the motor and by changing pulleys in the transmission system The second way of changing flicker frequencies consists in the use of different striped screens There are three screens available with 10, 20, and 40 stripes each The cylinders with a greater number of narrower stripes will give a higher flicker frequency with a slow speed of rotation Since the quietness of motion is essential for the precise judgment of threshold reaction, efforts were made to keep the number of revolutions of the screen always as low as possible

The striped screen is viewed by the fish against a white reflecting surface This is a hollow 45° cone made of sheet metal and painted with zinc oxide The cone

is illuminated from underneath. The light comes from a source (500 watt concentrated filament lamp) which can be placed at different distances on an optical bench. For experimentation three fixed positions of the source were chosen from the diffusing screen (*D*) at the end of the optical bench. Behind this screen there is placed a diaphragm which controls the area of the radiating surface of the diffusing screen. The light then falls on a mirror which is inclined at an angle of 45° and reflects through the glass top of the table on to the reflecting cone. The light intensities falling on the eye of the fish are measured by means of a Macbeth illuminometer. By means of the diaphragm and the different distances of the source from the diffusing screen the brightness can be varied over 4 logarithmic units. For each position of the light source and for the different diaphragm openings, calibration curves are plotted from which during experimentation the intensity values for a given diaphragm opening can be read with sufficient accuracy. The intensity range thus secured is not large enough to determine a complete flicker curve for the eye of *Lepomis*. For higher intensities which were desirable we had to use a 1000 watt lamp instead of the 500 and in some cases we had to use an additional source consisting of another 1000 watt lamp which was placed at a slight angle to the optical axis. This source then was kept constant and the one on the optical rail varied. By these means we were able to secure brightnesses sufficiently high to cause the fish to react at flicker frequencies as high as 50 per second. For intensities lower than the ones which could be obtained with the 500 watt lamp at the far end of the optical bench and by the diaphragm we used another source consisting of a 100 watt bulb at a fixed position and placed in front of it Eastman Kodak "neutral" filters of different transmissions. The neutral filters were calibrated with a Martens polarization photometer. First a 1/10 filter was calibrated, then keeping the 1/10 filter in place, a 1/100 filter is put into the path of the other beam of the photometer. The difference between the two is then about 1/10, which can be read with the necessary accuracy on the scale of the photometer head. In the same way a 1/1000 filter is calibrated against the 1/100 and finally a 1/10,000 filter against the 1/1000. For each filter in the light path a calibration curve for the diaphragm is plotted. The intensity range is thus extended over another 4 logarithmic units so that a total range of 9 logarithmic units can be covered.

For experimentation we selected 12 animals which gave rather precise reactions to moving stripes. These were among a great number of individuals which we obtained from the Boston city aquarium. The 12 selected animals were kept in separate glass jars. For tests they were transferred into culture dishes 10 cm in diameter and 5 cm in height. Each dish was filled with 250 cc of clean tap water which was at room temperature before the fish were put into it.

Before experimentation the fish were kept in darkness for at least 2 hours to secure a sufficient degree of dark adaptation. The first animal then is placed on the glass top of the table and left in the dark for a short time so as to avoid any interference with our first reading by the shock produced by the transportation. Then the stripe system is set into motion at a certain flicker speed, which was

previously fixed. The light is turned on while the diaphragm is completely closed. By means of a gear transmission the observer is able to open the diaphragm slowly while watching the animal. As soon as a small amount of light strikes the fish it shows a slight "shock reaction" by moving backward, it then stays quiet. This "shock response" is certainly not due to the flicker, since it can also be obtained with the stripes quiet. The fish's reaction to the moving stripes at threshold intensity consists in a sudden motion of the body in the direction in which the stripes are moving. For this threshold reaction the reading of the scale of the diaphragm is noted down which later on can be translated into the corresponding light intensity. At low flicker frequencies, *i.e.* 3 to 10 stripes per second passing in front of the fish's eye, the animal usually follows the stripes. It often stays right in the middle of the tank and turns around like a galvanometer needle keeping the same stripes in its field of vision. At speeds up to 15 or 20 flickers per second the fish stays to the wall of the glass jar and moves in circles. If the flicker frequency is above 20 per second the reaction mostly consists after a short forward motion, in swimming backward. This backward motion becomes faster with increasing flicker frequency. It therefore seems as if the fish moves in such a way as to make the flicker disappear by moving just fast enough against the motion of the stripes so that the visual field looks as if it was evenly illuminated.

Even while the kind of reaction can be quite different in nature the threshold illumination for initiation of response can be determined with accuracy. We usually took three successive readings with each fish at a given flicker frequency. Such a set of successive measurements gives approximately the same reading in each case. For each flicker frequency our 12 animals were tested and the threshold intensities for response recorded. The intensity values obtained for the whole group agree among each other fairly well. The animals are of the same species and of one strain. It therefore seemed justifiable to treat the data *en masse* for plotting a flicker curve for the eye of *Lepomis*.

III

The data for critical flicker frequency for the eye of the sun-fish are presented in Fig. 2, where we plot critical flicker frequencies against the logarithms of threshold intensities. The points of the curve are mean values of the observations taken on our 12 animals. It is at once apparent that the curve is made up of two distinct parts. For flicker frequencies between 3 and 10 flickers per second the slope of the central part of the curve is 1.3. Above flickers of 10 per second the curve takes a different slope up to about 43 flickers per second. The slope of this part is 16.8. Above 45 flickers the curve shows a bend to the right and comes to a maximum level a little above 50 flickers per second.

The two distinct parts of the curve indicate that we meet in the eye of the fish a dual visual system. The lower portion of the curve represents the function of the rods up to intensities a little above $1/100$ millilambert. At higher intensities the cones come into play.

If we attempt to compare the flicker data for the fish's eye with those obtained for the human eye (Hecht, Schlaer, and Verrijp, 1933-34) we have to take three points into consideration

1 The intensity range covered by the fish is considerably greater for the rod section of the curve than for man. The fish will react to the

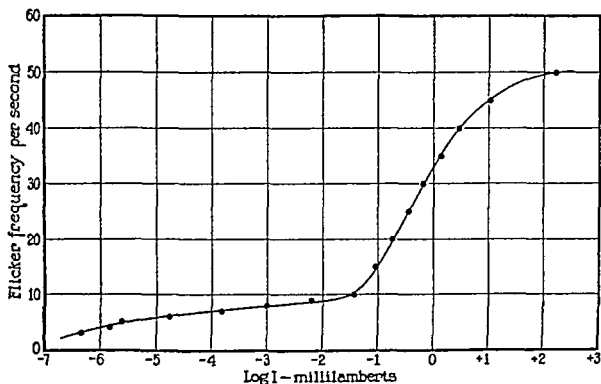


FIG. 2. Relation between critical flicker frequency and illumination for the eye of the sun fish. The points represent mean values of threshold intensities for 12 fish tested at different flicker frequency.

moving stripes at intensities which are lower than necessary for the human eye to recognize flicker at all. The curve for the fish extends so far that at the very lowest intensities it is hardly possible to see the fish or to decide whether the fish was reacting to the flicker even before the light intensity was sufficient for the observer to see the fish. The fish is certainly capable of recognizing flicker at intensities 2 logarithmic units below that required for man.

The slopes of the rod sections of the curve for the human eye and

for the fish are quite different and are hard to compare. The human curve shows a plateau in that section which covers about 3.25 logarithmic units, whereas the curve for *Lepomis* shows a steady rise. Hecht, Schlaer, and Verriyp (1933-34) have given a slope value between 4.5 and 5 for the rod part of the human eye, for the fish it is 1.3.

2. The transition from rod to cone vision occurs for the human eye at about -2.5 of the log I scale¹ and at a flicker frequency between 9 and 10. For *Lepomis* the transition occurs at the same intensity and at the same flicker frequency.

3. For white light the cone sections of the human and the fish curves are quite different in slope. For the human eye the values average around 11, whereas for the fish we find a slope of 16.8. The maximum flicker frequency which can be perceived by the human eye varies between 45 and 53 per second. The maximum for *Lepomis* is very similar. Some of the animals will not give a clear response at 50 flickers per second, some will still react at frequencies slightly over 50. It has to be stated, however, that the response at illuminations higher than 100 millilamberts is not as clear as at lower intensities. It seems therefore probable that very bright illuminations might have some injurious effect which prevents proper reaction. For the human eye there is found a drop in flicker frequencies at intensities close to 1000 and over 1000 photons. With our present experimental arrangement we are not able to obtain light intensities as high as those used for investigations on the human eye. We therefore are not able to state at present whether the same drop in critical flicker frequencies could be found at very high intensities for *Lepomis*.

It remains to compare the flicker curve for *Lepomis* with that for the bee (Wolf, 1933-34), since for both organisms the same kind of reaction to moving stripes was taken for the determination of threshold reaction. The flicker curve for the bee is quite different, in two respects. First, the slope of the curve is very steep over the middle range, second, there is no evidence for the presence of a dual visual system. We might deduce from these facts that the ommatidial mosaic of the bee's eye is not divided into two distinct groups of

¹ Hecht's measurements are given in photons. On converting his intensity values into millilamberts the change is found to be insignificant for the purposes of this comparison.

receptors of which one is acting in dim light, like the rods, and the other in bright light, like the cones. We probably meet only one system of receptor elements which covers the whole visual range.

The relationship between threshold light intensities and flicker frequencies suggests that there exists a connection between the effect produced during each period of excitation by light and the duration of the dark period within each complete flicker cycle. Reaction to flicker ceases at the moment when, at a given intensity of illumination, the duration of the light and the dark periods becomes shorter than necessary to unbalance the photochemical receptor system, which would cause a response. At this instant we encounter a steady state condition in which during exposure to light the amount of photolytic products required to initiate an impulse, is below threshold concentration, and where during the dark period the time is shorter than necessary to rebuild a sufficient amount of photosensitive material which could be acted upon by the next flash of light. For the stationary state condition a photochemical equation has been derived. If we attempt to fit the flicker data for *Lepomis* to the stationary state equation we obtain a very good fit for the rod part of the curve, and also for the cone part. Since the general theory of stimulation by intermittent light has been discussed recently in a number of papers (Hecht and Wolf, 1931-32, Hecht, Schlaer, and Verriyp, 1933-34) it is not necessary to give a theoretical interpretation of the flicker data secured with *Lepomis*.

SUMMARY

The sun fish *Lepomis* responds to a moving system of stripes by a motion of its body. By changing the velocity of motion of the stripe system different flicker frequencies can be produced and thus the relation of flicker frequency to critical intensity of illumination can be studied. Threshold illumination varies with flicker frequency in such a way that with increasing flicker frequency the intensity of illumination must be increased to produce a threshold response in the fish.

The curve of critical illumination as a function of frequency is made up of two distinct parts. For an intensity range below 0.04 millilambert and flicker frequencies below 10 per second, the rods are in function. For higher intensities and flicker frequencies above 10,

the cones come into play The maximum frequency of flicker which can be perceived by the fish's eye is slightly above 50 per second

The flicker curve for the eye of *Lepomis* can easily be compared with that for the human eye The extent of the curve for the fish is greater at low illuminations, the fish being capable of distinguishing flicker at illuminations lower than can the human eye The transition of rod vision to cone vision occurs for the fish and for the human eye at the same intensity and flicker frequency The maximum frequency of flicker which can be perceived is for both about the same

CITATIONS

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ON THE VARIABILITY OF CRITICAL ILLUMINATION FOR FLICKER FUSION AND INTENSITY DISCRIMINATION

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I

The properties of events constituting stimulation or excitation are established by measurement of aspects of excitation as functions of the exciting energy. This involves the averaging of measurements of response, or of energies associated with a chosen index of responsiveness, or of both. The real meanings of such averages may be quite different in the several instances, with regard to the uses to which it may be desired to put them. The averaging process itself may not be overt, but may be concealed in the method whereby the recorded observations are obtained.

When indices of the dispersions of the measurements entering into such averages can be given, additional information may be secured which is of distinct utility. It can provide criteria for the homogeneity of the assemblage of data. It also makes possible a functional analysis of variability of organic performance (*cf* Crozier, 1935). And these indices of dispersion appear to have a very important bearing upon the interpretation and the use of the data of sensory discrimination, they have of course long been employed, in a sense, in connection with the so called psychometric functions, but it seems without adequate realization of certain of their properties. In the customary type of experiment, for example, two intensities of an exciting variable are judged just equal, but it is of course their *effects* which are seen not to be detectably different. The distinction is real and necessary, it is required to obtain independent but congruent information concerning the relation between intensity and effect before the basis for the distinction of two effects can be understood. A larger discussion of this matter will be undertaken elsewhere. We wish to deal now

with certain empirically determined properties of the data upon which the curves of some visual functions are based. These serve to extend the variety of cases in which the laws of organic performance are equally evident in the variation of the measurements and in the means (*cf* Crozier, 1929, Crozier and Pincus, 1927-28, 1929-30, Wolf and Crozier, 1932-33, Crozier, 1935). The data to be discussed have in common that they depend upon the comparison of alternately disposed stripes of equal width illuminated by two intensities, one of which may be zero. They also have in common that they are based upon constant numbers of observations in each series. It is important to discover if the analysis of objective measurements possessing a certain homogeneity of character shows their properties to be similar to those of subjectively based judgments or decisions in similar tests. The comparison of findings and relationships in the present inquiry with those which result from the examination of properties of subjective judgments of just noticeable difference may be expected to provide an interpretation of the nature of such judgments as they are functionally dependent upon variables experimentally controlled.

II

When intensity discrimination is measured in the eye of the bee (Wolf, 1932-33*a, b*) by using a moved background of alternating equally wide stripes respectively illuminated by intensities I_1 and I_2 , a threshold response is obtained from the bee when $(I_2 - I_1)$ has a certain mean value for each magnitude of I_1 and for stripes of a given width. $I_2 - I_1$ defines a value of ΔI , the "just detectable difference of intensity" characteristic of I_1 (or of I_2). It is sometimes overlooked that ΔI is described not only by its mean value, but also by the properties of the frequency distribution of the measurements from which average ΔI is computed. From the standpoint of demonstrating the propriety of deriving quantitative laws for the performance of biological systems, in the face of the variability which all properties of organisms manifest, it has been important to demonstrate that the variability of measurements of performance, for example of ΔI regarded as a measure of excitability, is lawfully related to the independent quantity I responsible for the effects measured under the given conditions (*cf* Crozier, 1935). It is also to be demonstrated

that the variability of such measured indices as ΔI must be carefully considered in the light of the manner in which mean ΔI 's emerge from the experimental procedure, before fully effective theoretical use can really be made of them

From the data secured with the visual response of the bee (Wolf, 1932-33 *a, b*, 1933-34) it was possible to show (*cf* Wolf and Crozier, 1932-33) that for intensity discrimination by the method described the curves obtained on plotting the "Weber fraction" $\Delta I/I_1$ against $\log I_1$ are of the same form as those given by $\sigma_{(\Delta I/I_1)}$ against $\log I_1$, for each width of stripes employed (*i.e.*, for the various visual acuities called upon), σ = standard deviation of the mean, the number of observations entering into each average is the same for each value of I . This says that one can write

$$\delta(\Delta I/I_1) = \phi(I_1) = k\delta(\sigma_{\Delta I/I_1})$$

By the procedure employed in obtaining these data I_1 is fixed when each value of ΔI is obtained (When the method used involves simultaneously changing both I_1 and I_2 , but keeping the ratio constant, it may be impossible to undertake a satisfactory analysis of the observed variations in sensitivity. The reasons for this will be considered in detail elsewhere.) Hence $\sigma_{(\Delta I/I)} = \sigma_{\Delta I/I_1}$, and since $\sigma_{\Delta I} = \sigma_{I_1}$, we have $d(\Delta I/I) = kd(\sigma_{I_1}/I_1)$. In other words,

$$\Delta I = k\sigma_{I_1} + \text{const}$$

This is directly illustrated in Fig. 1. The slope of $\log \Delta I$ as a function of $\log \sigma_{I_1}$ is constant, and is not distinguishably different from 1. The position of the graph on the coordinate grid differs slightly from one lot of bees to another in experiments made at different times, but is the same for the same lot, and k is independent of I and of the width of the test stripe. In Fig. 1, $\log P.E._{\Delta I}$ is given as a function of $\log \Delta I$. $P.E._{\Delta I} = 0.6745\sigma_{\Delta I}$, and is the same as $P.E._{I_1}$. For Series A (Wolf, 1932-33 *a*), first set, there is a slight apparent difference in slope from that given by the measurements with other widths of stripe, the experiments were done a year before the others, and are shown separately. The upper limiting line has a slope of 1. Error in the setting of I_2 at very low values might well make $P.E._{I_1}$ there appear too high. The breadth of the band should be constant, since $\sigma_{P.E._{I_1}}$ should be

proportional to $P E_{I_2}$, in Series *A* this seems not to be the case, at the upper end of the graph the breadth is that given by the other measurements, it is to be presumed that a larger number of determinations in this series would show the band broader at the lower end. A second set of tests (Wolf, 1932-33 *a*), also shown in the left hand graph (Fig 1) supports this view. The relationship of ΔI to σ_{I_2} is independent of the width of the stripes in the test pattern.

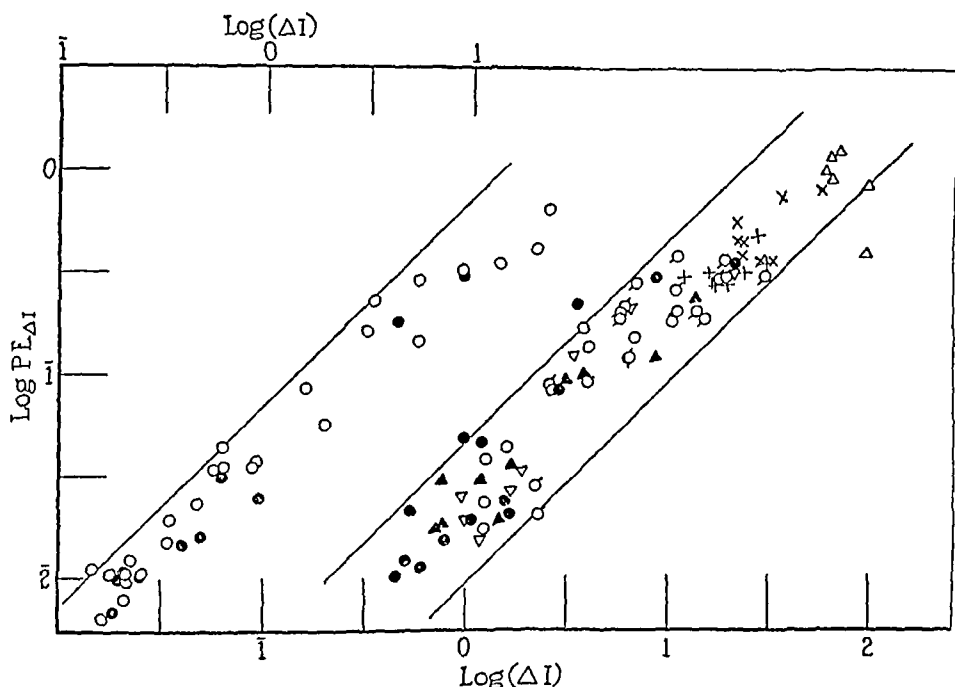


FIG 1 $\log P E_{\Delta I}$ as a function of $\log \Delta I$, from experiments on intensity discrimination by the bee (data in Wolf, 1932-33 *a, b*, Wolf and Crozier, 1932-33). Data from one series, the earliest (Wolf, 1932-33 *a*, "Series *A*") are shown separately at the left, see text

The proportionality of ΔI to σ_{I_2} (or $\sigma_{\Delta I}$) has a very interesting bearing upon the problem of intensity discrimination. It is not our purpose to enlarge upon this at the moment. But it is of value to indicate that the rule is obeyed under conditions such that the bee's capacity to respond is changed, not by altering I , but by employing the animal's changing photic sensitivity during dark adaptation. In this case, the magnitude of I required to elicit a threshold response declines with

the length of time during which dark adaptation has taken place (Wolf and Zerrahn Wolf, 1935-36*a*) The method here consists in having stripes (of maximum usable width) alternately illuminated by intensity I and dark, the same threshold response is employed, and values of I are found which give the threshold response at times t

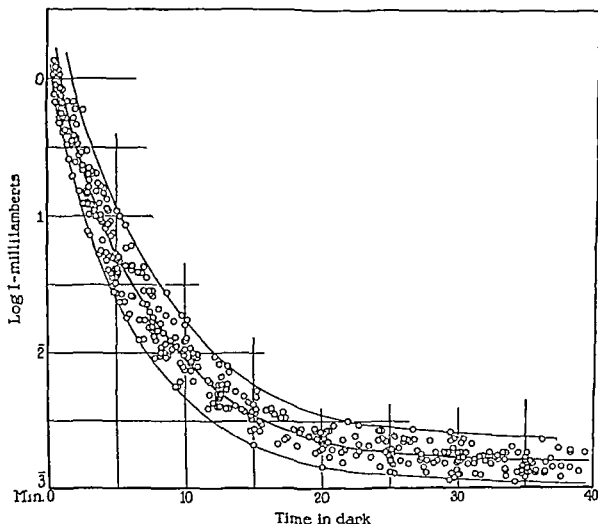


FIG 2 Dark adaptation in the bee individual determinations of photic intensity required to produce reaction to a stripe system after various periods in darkness subsequent to light adaptation see text

The single readings obtained in this way are scattered in a band with definite enveloping margins. When $\log I_1$ is plotted as a function of time in the dark the curve is not hyperbolic (Fig 2), but the line of central tendency corresponds quite precisely with that given by the equation

$$-\log (\log I_1/I_f) = kt + \text{const}$$

where I_t = mean I and at time t and I_l = mean least intensity (Wolf and Zerrahn-Wolf, 1935-36 *a*) The lowest intensity evoking response at time t is labelled I_a , the highest I_b . These must be estimated, in the

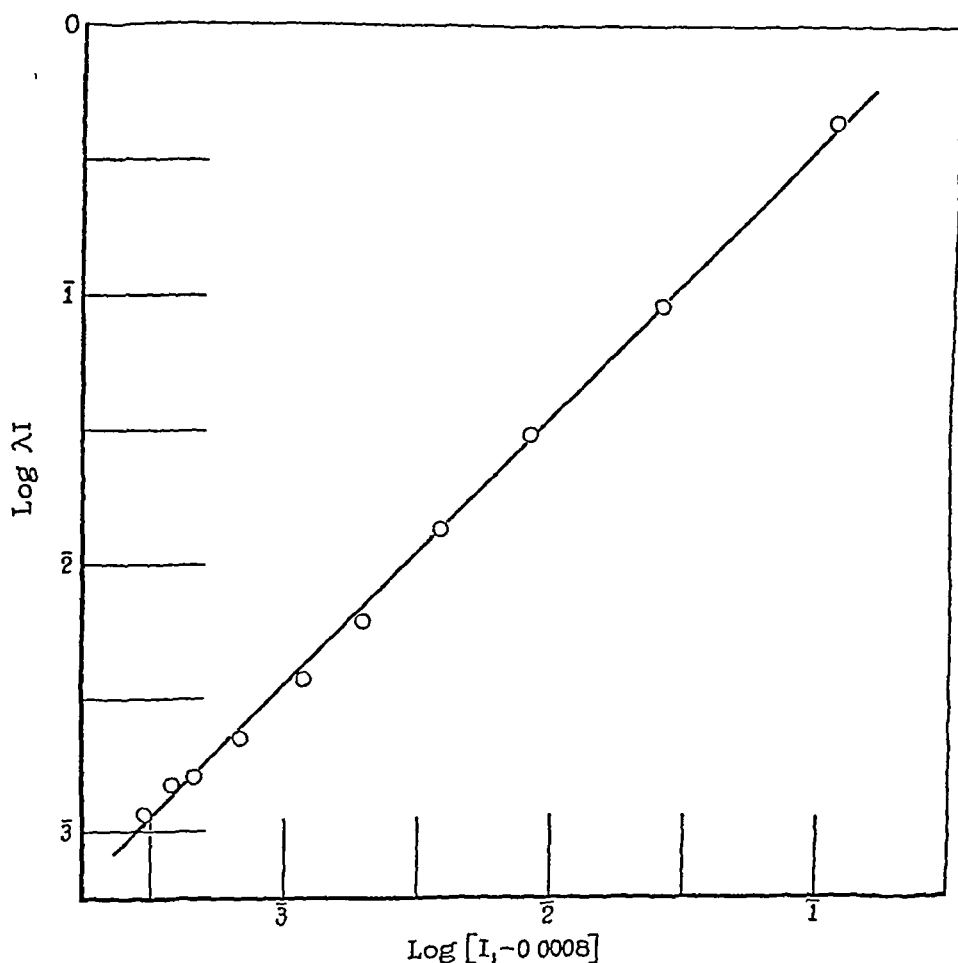


FIG 3 $\log \lambda I$, the latitude of variation of intensity required for threshold response to a stripe system at various levels of dark adaptation, as a function of $\log I_1$, the lowest intensity giving the response at the respective levels of dark adaptation (*cf* Fig 2), $\log \lambda I$ is directly proportional to $\log (I_1 - \text{threshold } I)$, and the slope = 1

nature of the case, from the marginal envelopes fitting the array of plotted points. The latitude of variation, at fixed time t is $(I_b - I_a) = \lambda I$. The method of obtaining the threshold response is such that λI

corresponds in meaning to σ_{I_1} in the tests already considered, and in the experiments on visual acuity (mentioned subsequently),—provided equivalent care has been employed in making each separate measurement, the consistency of the behavior of λI makes it clear that this requirement has been met. λI is directly proportional to I_a , so that

$$\log \lambda I = \log [I_1 - 0.0008] + \text{const}$$

(see Fig. 3) The constant 0.0008 corresponds to the fact that there is an upper limit of excitability, and consequently an asymptotic lower limit of I capable of evoking the response with stripes of the width used (*cf* Fig. 2). Since the distribution of I is "normal," $(I_a + I_b)/2$ corresponds to the mean value of I ($= I_m$) at each t , hence I_m is directly proportional to λI . In this respect the array of individual determinations is comparable to that seen in curves of frequency or speeds of vital activities as a function of temperature (Crozier and Federnghi, 1924–25, 1925, Crozier and Stier, 1924–25, Crozier, 1929, 1935). In the present case, the latitude of variation of I is directly proportional to I . This indicates, among other things, that the variation in I is not fundamentally caused, at constant t , by fluctuations in the velocity of dark adaptation due to its being a complex process, otherwise λI could scarcely follow the same law as I_m . The distribution of the determinations in a band of this sort shows the latitude of distribution of the probabilities, at each value of t , that a given bee will exhibit the index response.

If two values of I , I_1 and I_2 , were to be distinguished as just detectably different, at various levels of t , on the basis of a uniform statistical criterion applied to the system illustrated in Fig. 2, namely in terms of their abilities to call forth just threshold response at different levels of receptivity, it is clear that, I_1 being fixed and I_2 being adjusted,

$$\Delta I = I_2 - I_1 = k\sigma_{I_2} + \text{const}$$

from what we have just seen,

$$\sigma_{\Delta I} = \sigma_{I_2} = k I_2 + \text{const}$$

and consequently ΔI is directly proportional to I_2 . It is clearly difficult to get a " ΔI " by this means directly, but its significance is impor-

tant The understanding of this particular situation is made easier by the fact that one is not required to compare the magnitudes of a series of effects produced by each of two chosen intensities, but merely the probability distributions of occurrences in two sets of one constant effect—namely threshold response, at times t_1 and t_2 (for an analogous case, see Crozier and Pincus, 1931–32 *b* (p 244, *footnote*), Crozier, 1935)

We may also consider that for threshold response in these tests I_2 must be just distinguished from $I_1 = 0$ The visual angle of the stripes is kept constant, but the excitability increases with time in the dark Hence the data give us values of $I_2 - 0 = \Delta I$ at various levels of excitability, ΔI is then $= I$, and $\sigma_{\Delta I}$ is directly proportional to I In either case, the result is the same as in the consideration of the data on intensity discrimination

The limiting case of intensity discrimination is supplied by the measurements of visual acuity Here (Hecht and Wolf, 1928–29) the width of the stripe is varied, the stripes are alternately illuminated and dark, for each width of stripe the illumination is determined which just evokes threshold response when the background is moved It is not a question of matching two intensities on the basis of their effects, I_1 is zero, and $I_2 = \Delta I$ Clearly, for this case also $\Delta I/I_2 = 1$ In terms of the preceding discussion we must expect to find $\sigma_{\Delta I}$, here the same as σ_{I_2} , to be directly proportional to I This is best tested by plotting $\log \sigma_I$ as a function of $\log I$, σ_I was computed from the tabulated values of $\log I$ in the paper by Hecht and Wolf (1928–29), σ_1 is used in this case, since n is not the same throughout It turns out that

$$\sigma_{I_2} = (I + 0.040)/5.02,$$

as shown in Fig 4 The curve does not pass through the origin When $I = 0$, $\sigma_I = 0.0079$ This means that there is a residual variation of reaction, reflected in the variation of the settings of I , which is not fully accounted for by the influence of I upon the threshold reaction This may well be due to the effect of transition of ommatidia from the illuminated to the “dark” state (or the reverse) (*cf* Wolf, 1933–34, Wolf and Crozier, 1932–33, Wolf and Zerrahn-Wolf, 1934–35) In the visual acuity tests and in other tests by this method

involving the appearance of a threshold response, the frequency of such transitions is low, and small changes in this frequency are without effect upon mean I (*cf* Wolf, 1932-33*a*), but variations in the effect of this factor would appear independently of changes (fluctuations)

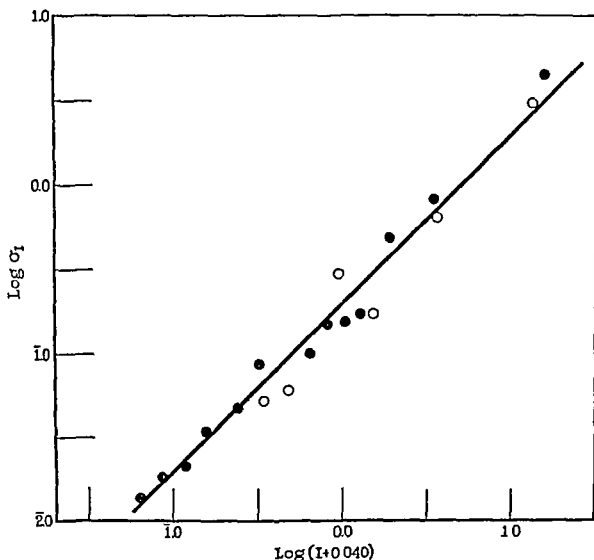


FIG. 4. $\log \sigma_I$ as a function of $\log I$ from data on visual acuity (bee) see text. The slope is 1. Data in Hecht and Wolf (1928-29). From the line drawn,

$$\log \sigma_I = \log (I + 0.040) - 0.70$$

Solid circles: normal eyes; open circles, eyes opaqued on the anterior half

in the effectiveness of I , and (since this factor is relatively without effect upon mean I) should be detected as a constant value of σ_I , their presence being perceptible only through the measurements of I . The result given in Fig. 4, namely that $\sigma_{I_2} (\equiv \sigma_{\Delta I})$ is directly proportional to $I_2 (\equiv \Delta I)$, is the one already found in the data of intensity

discrimination (Fig 1) The direct proportionality of σ_I to I makes it possible to fit exactly the same curve for visual acuity to σ_I and to I This is shown in Fig 5, the curve for σ_I is shifted laterally (by 0.720 units)

In the visual acuity tests a decrease in the width of the stripes is made up for by an inverse change in I so that a threshold excitation is

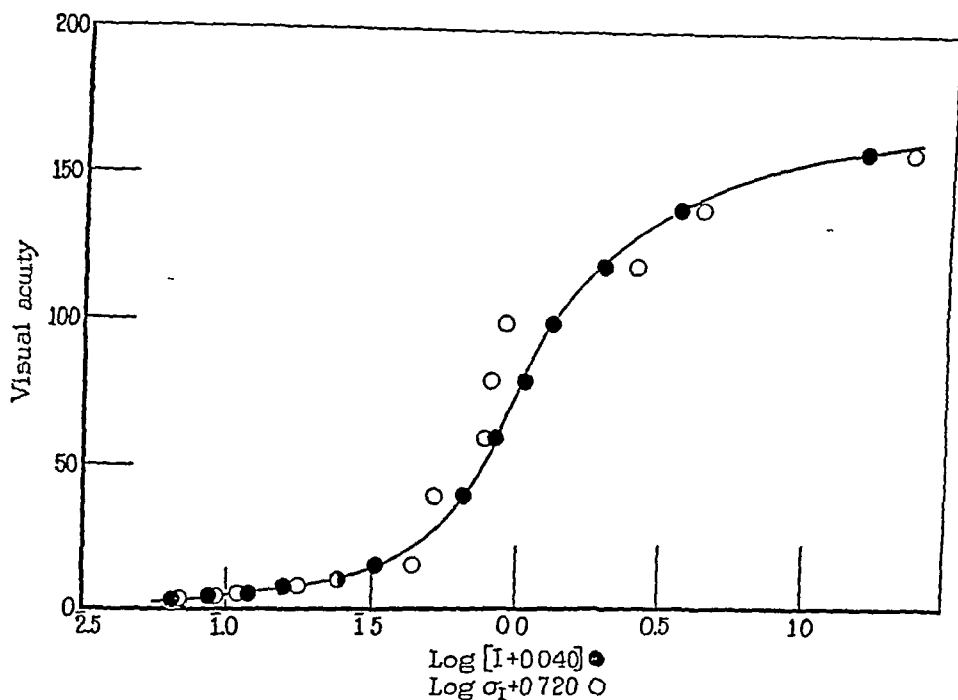


FIG 5 The curve of visual acuity for the bee, expressed as a function of $\log (I + 0.040)$, also describes the relation of visual acuity to $\log \sigma_I$ (The fit is satisfactory in terms of the plus and minus deviations at fixed values of visual acuity, the deviations, in other words, have to be considered in the *abscissa* direction, and they are of about the same magnitude all along the curve)

induced by movement of the visual field It has been shown with the bees that the phototropic effects of two illuminated fields of similar contour but differing in area and in illumination become equal when the product of area by intensity is the same for both fields (Wolf and Zerrahn-Wolf, 1934-35) Moreover, to a flickered field, below the critical frequency of interruption, the reaction is greater than to a non-flickered field of the same mean intensity It might then be

assumed that the total stimulation at the threshold for response must be taken as proportional to I , to V , and to $1/V$, where V is the visual angle subtended by a stripe, and $1/V$ measures the frequency of transitions from dark to light. Hence the stimulation should be proportional to I . Variations in the threshold sensitivity would be reflected in variations of I . But since the same fraction of the field is illuminated with all stripe widths, namely 50 per cent, the total number of receptors functioning must be higher if I is higher, assuming a frequency distribution of intrinsic thresholds (Hecht and Wolf, 1928-29). But if a larger number of receptors is involved when I is higher, a lower frequency of excitation should suffice. One way out of this difficulty is to recognize that the threshold excitability of a receptor shows cyclic changes, but that the *average* excitability of all ommatidia is the same. It has not been found possible satisfactorily to rectify the curve connecting visual angle with I for threshold response (in the bee) in such a way as to show that visual angle and I are related reciprocally, that is, $(V + m)(I + n) \neq \text{const}$, where m and n are constants. But when the area of the eye is reduced by painting over a portion of the ommatidial surface, the intensity required for response at a given visual acuity is increased. The same general rule, however, holds as with the normal eye, σ_I is again directly proportional to I (Fig. 4), σ_I might be expected higher, due to variations in the amount of eye surface blocked out in the different individuals, but the proportionality still to hold, this supplies an interesting check upon the differentiation between "experimental error" and variability by the method of expressing variation of I as a function of the measured intensity. It may be that the stimulation required for just detectable response is not the same for all levels of I , with this method. We know that the threshold response used as an indicator of excitation differs in at least one respect as threshold I increases, namely in that its sharpness and definiteness are augmented, but σ_I is greater as I increases, so that the mere relative sharpness of the response certainly cannot be held responsible for the change in σ_I . Our interest in these considerations is at the moment concerned with the essential equivalence of ΔI and $\sigma_{\Delta I}$, if the total excitation is the same at all levels of I for the index response, " $\Delta I/I$ " would necessarily be constant, but it does not appear obligatory to make this assumption.

The data here are entirely of the same sense as in the case of the curve of dark adaptation (Fig 3), there, different levels of excitability are attained by altering the level of the stationary state of light adaptation, in each case, ΔI is directly proportional to I , and $\sigma_{\Delta I}$ is directly proportional to ΔI

III

Flicker fusion depends upon the suppression of intensity discrimination For the bee the relation of frequency of intermittence of a light and its critical intensity for response to flicker has been investi-

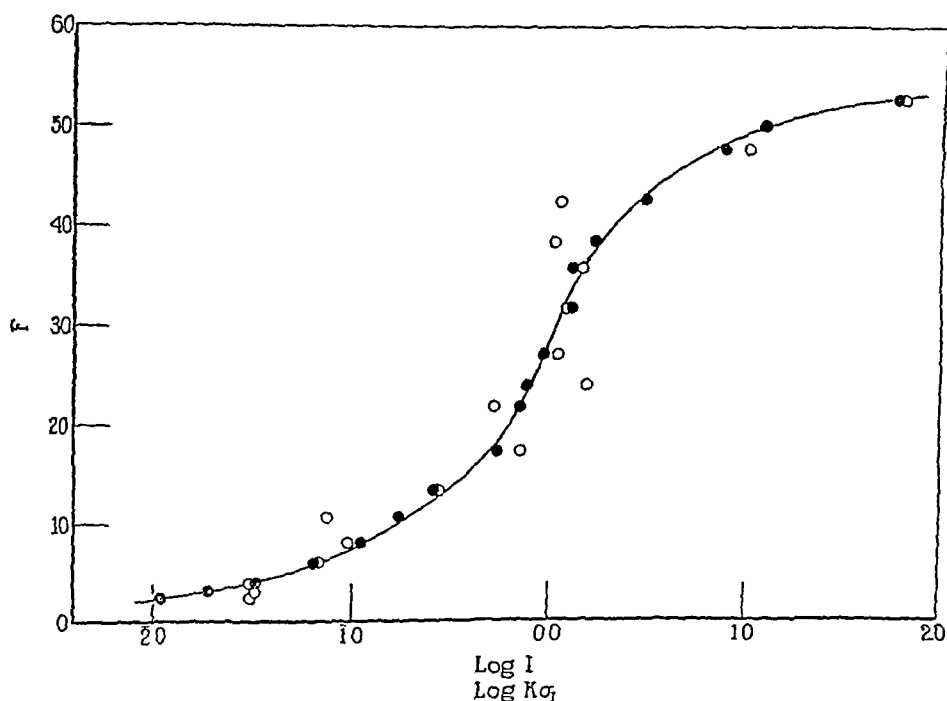


FIG 6 Flicker frequency F and mean intensity I for flicker fusion, in the bee (data from Wolf, 1933-34), solid circlets, I , open circlets, $k\sigma_I$

gated (Wolf, 1933-34) The experiment involves measuring values of I adequate to produce fusion of the effects of flashes at fixed frequencies The flashes are separated by equally long dark spaces Fusion is indicated by failure of a response otherwise given to the field of successive light and dark sectors For these measurements (cf Wolf, 1933-34, Fig 4)

$$\log \sigma_{I_2} = k \log I_2 + \text{const} ,$$

k is not certainly distinguishable from 1, hence

$$\sigma_I = mI_1$$

(A better fit is obtained by adding a small constant to I_2) As Fig 6 shows, the curve connecting frequency of intermittence (F) with mean critical illumination for response (I) is duplicated by the relation of σ_I to F . As in Fig 5, the *lateral* departures should lie within a band of constant width, if many determinations were available. Hence

$$d\sigma_I/dF = \phi(F) = k(dI/dF)$$

and

$$d\sigma_I/dI = K$$

This is of course the result already given for the data upon intensity discrimination, dark adaptation, and visual acuity, and is to be interpreted in a similar way. We may say that the two intensities distinguished when flicker is reacted to are I_1 ($= 0$) and I_2 , and again I_2 is to be formally identified with ΔI . If two mean intensities I_1 and I_2 were to be judged objectively as just significantly different, by means of the flicker curve, one of these intensities (I_1) being fixed, then

$$\Delta I = k(\sigma_{I_1}) = k I_1$$

and

$$\Delta I/I_1 = k$$

The experiment is not usually made in quite this way. We shall see in a later connection that if ΔI were to be determined by a comparison of mean values of F for two fixed magnitudes of I , the behavior of the ΔI as a function of I would be found quite different.

IV

By a method similar in essentials to that used for the bees a "flicker curve" has been obtained for the sun fish *Lepomis* (Wolf and Zerrahn Wolf, 1935-36 *b*). It has the general form found for the corresponding property of the human retina (Hecht and Verrijp, 1933-34 *b*) when $\log I_m$ is plotted as a function of F . It has a lower segment of flat slope in the range of intensities where rod vision is

exclusively or predominantly concerned. The upper, steeper segment corresponds to the rôle of cone vision at higher intensities. This division permits a special kind of test of the notions here developed with regard to the analytical significance of the variation of I in such determinations. The measurements were made as with the bees, by

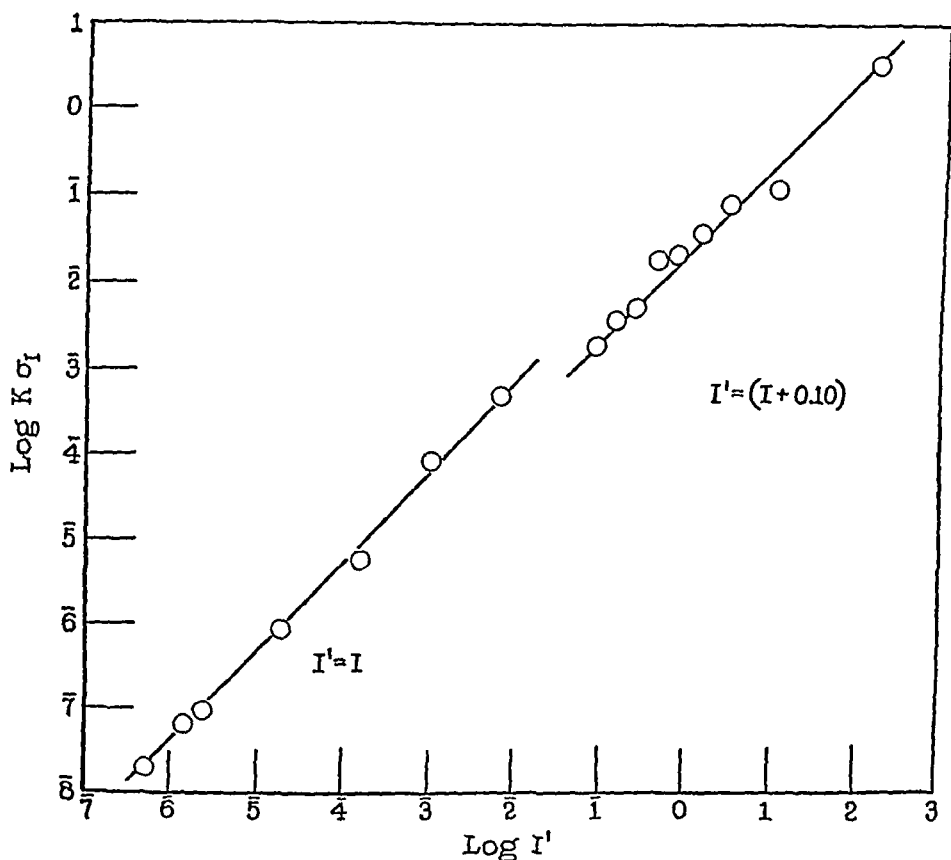


FIG. 7. $\text{Log } \sigma_I$ as a function of $\text{log } I$ for extinction of flicker with the sun-fish *Lepomis* (data in Wolf and Zerrahn-Wolf, 1935-36 b). The lower line refers to rod functioning, the upper to the cones, see text.

finding, for each of a number of fixed values of the frequency of intermittence (F), the mean value of I which just gave threshold response to a moving background of equal stripes alternately black and illuminated by intensity I . The technic has been described in detail (Wolf and Zerrahn-Wolf, 1935-36 b).

For the rod section of the curve,

$$\log \sigma_I = k \log I + \text{const}$$

and k is not certainly different from 1, in Fig 7 the line is drawn with slope 1.05, but the difference from 1 is probably not significant. For the cone section (Fig 7),

$$\log \sigma_I = \log (I + 0.10) + \text{const}$$

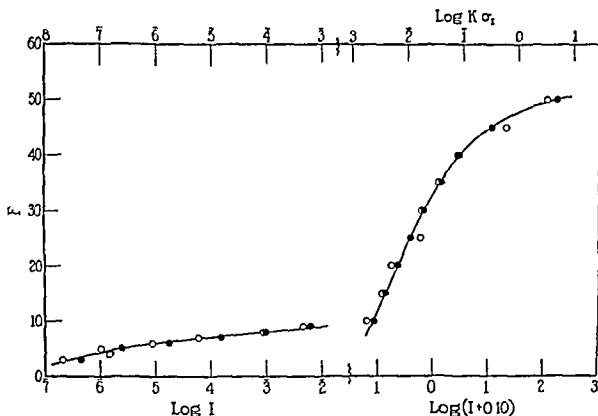


FIG 8 $\log I$ as a function of flicker frequency F , for rods (left) and cones (right) the curve of $\log I$ is the same as that for $\log \sigma_I$ (open circlets), when for the cones I is replaced by $(I + 0.10)$. See text

The constant 0.10 means merely (as in the case of the bee visual acuity data) that σ_I is directly proportional to I , but that the line does not go through the origin. Since the cone segment of the curve is superimposed upon the rod segment, σ_I for the cones has to be diminished by an amount characteristic of the full excitation of the rods at the threshold I for excitation of cones, arithmetically this is the same as adding a constant to I for this portion of the curve.

It follows that the curve fitting $\log I$ as a function of I , for the rods,

must also describe $\log \sigma_I$ as a function of F . The left hand graph in Fig 8 demonstrates this. For the cone portion, $d(\log(I + 0.10))/dF$ must $= d(\log \sigma_I)/dF$. The right hand graph in Fig 8 shows that the agreement is excellent.

The relationship found between I and σ_I for extinction of flicker is consistent with the results of intensity discrimination tests (p 505). When the flickered light is just reacted to (threshold I), it is a question of "recognizing" I_x (in the light sectors) as just different from $I_x - \Delta I_x$. $I_x - \Delta I_x$ may be regarded as determining the apparent effect of the dark sectors. Here we must deal with I_x as the mean apparent intensity of the flickered light at the point of extinction of flicker (equivalent to the point of extinction's just failing). Whether this means that for the sun-fish the field is then one of continuous brightness corresponding to I_x is a question we need not discuss. At flicker frequencies above the critical, Talbot's law holds (*cf* Hecht and Wolf, 1931-32). Apparently no measurements exist in the literature to show whether it holds at the critical frequency for fusion (or at the critical illumination), a matter which should be tested. We may assume that $I_x = n I$, as a first approximation. We have learned that $\Delta I = k\sigma_{I_x}$, for discrimination between I_1 and I_2 when I_1 is fixed and I_2 variable. Here, I_1 and I_2 both vary, although not independently, $I_2 = I_x$, $I_1 = I_x - \Delta I_x$. Then $\sigma_{\Delta I_x} = k'\Delta I_x$. From the intensity discrimination case (p 505) we also know that $\Delta I = k\sigma_{I_x}$, hence $\Delta I_x = k\sigma_{I_x}$, and since I_x is assumed $= n I$, $\Delta I_x = k''\sigma_I$. From the data we learn that $\sigma_I = K I$, ΔI_x is directly proportional to I .

v

Facts of the type here collected raise some curious problems of "curve fitting". The curve relating $\log I_m$ to F for the vertebrate retina (man Hecht and Verriyp, 1933-34*a*, sun-fish Wolf and Zerrahn-Wolf, 1935-36*b*) contains two portions or segments, referring to the functional involvement of rods and of (rods and) cones respectively. These curves define the relationship $d \log I/dF$, where F is (in the cases we have considered) the independent variable. For a given value of F , $\Delta I_x/\sigma_{\Delta I_x}$ is constant, and obtains for all values of F under the given conditions (as to width of stripes, etc.) at the point where the flickered light is just not reacted to. We have seen that $d \log I = d$

$\log \sigma_{\Delta I_x}$ hence $d \log I/d F$ is equivalent at each value of F to $d \log \sigma_{\Delta I_x}/d F$, assuming that $I_x = k I$. If we take the data as they stand in a table of F , I , and σ_I we find that for the cones $\log \sigma_I$ as a function of $\log I$ is very nearly rectilinear, with a slope of about 0.81 (Fig. 9). We have already seen that a more reasonable treatment shows that $\log \sigma_I$ is directly proportional to $\log (I + 0.10)$ with a slope of 1, but the other formulation might easily be employed. It means that to a

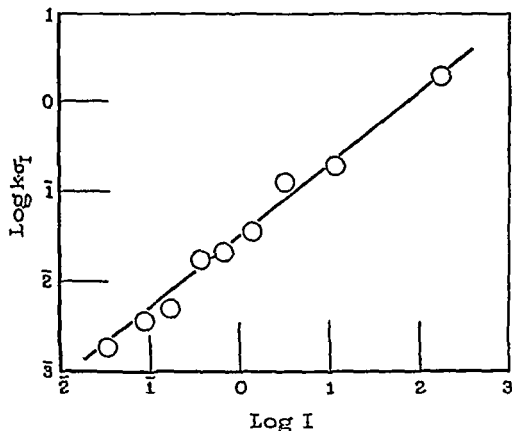


FIG. 9 $\log \sigma_I$ as a function of $\log I$, cone portion of the flicker extinction curve (*Leptomis*) uncorrected for the rôle of the rods. The relationship is nearly rectilinear, with a slope of 0.81.

very fair approximation $d \log \sigma_I = n d \log I$. In terms of average I secured by this method, a significant increase in I , due to an increase of F , will be such that $d \sigma_{I_x}/d I$ is really constant, but σ_I/I^n will appear constant, with $n = 0.81$. Therefore in fitting the cone curve of intensity discrimination to the data for critical illumination *versus* flicker frequency, I will seem to be in effect substituted for by a quantity (ΔI or $\sigma_{\Delta I}$) which is very nearly proportional to I^n . The suggestion is that the presence of an exponent such as n in the fitted curve (*cf*

Hecht and Verrijp, 1933-34 *b*), may thus result merely from the fact that the cone portion of the curve is added to the rod portion and that σ_{IC} then contains the σ_{IR} associated with maximal rod function, this has formally the effect of making σ_{IC} seem proportional to I^n , in a way which is really accidental

A related question concerns the form of the connection between (1) mean critical illumination for fusion of effects of flicker at fixed frequencies and (2) mean critical frequency of intermittence at fixed illuminations. It can be deduced from data of the sort we have considered that the two curves will not be identical. Let us take one example, the rod portion of the $F - I$ curve for *Lepomis* (Wolf and

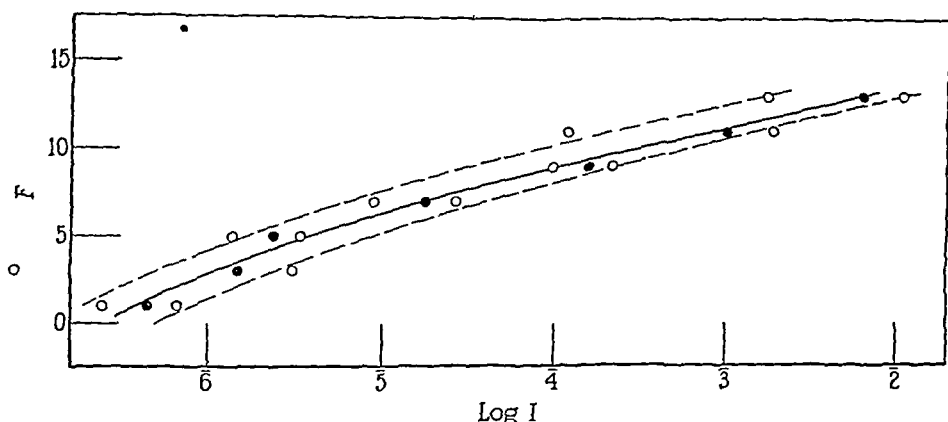


FIG 10 $\log I_m$, and $\log I_m \pm 10 \sigma_{I_m}$, for the rod portion of the flicker extinction curve of *Lepomis* ($10 \sigma_{I_m}$ is used merely for convenience in this case)

Zerrahn-Wolf, 1935-36 *b*) In Fig 10, $\log (I \pm k\sigma_I)$ is plotted as a function of F . The distribution of measured I 's at fixed F , measured by σ_I , gives the distribution of the threshold effects produced by intensity, at each value of F . The envelopes of these limits ($\pm\sigma_I$) enable us to define the distribution of the threshold effects due to varying F at fixed levels of I . The experiment made in this way will be considered in another paper. It is obvious, however, that σ_F will follow a different law from that observed for σ_I , and that mean F 's at fixed $\log I$'s will give a curve detectably differing from that of \log (mean I) at fixed F 's. (The problem is formally identical with the statistical one of fitting regression lines, the two problems need not

be the same from the standpoint of interpretation) The importance of these considerations may be considerable, since they appear to apply quite generally

SUMMARY

From the data of experiments with bees in which threshold response is employed as a means of recognizing visual discrimination between stripes of equal width alternately illuminated by intensities I_1 and I_2 , it is shown that the detectable increment of intensity ΔI , where $\Delta I = I_2 - I_1$, is directly proportional to σ_{I_2} (I_1 being fixed) From tests of visual acuity, where $I_1 = 0$ and the width of the stripes is varied, $\sigma_{I_2} = kI_2 + \text{const}$, here $I_2 = \Delta I$, and $\Delta I/I_2 = 1$ When the visual excitability of the bee is changed by dark adaptation, $\lambda I \equiv k\Delta I$ ($= k'\sigma_{\Delta I}$) $= k''I + \text{const}$ For the measurements of critical illumination at threshold response to flicker, σ_{I_2} ($= \sigma_{\Delta I}$) $= kI_2 = k'\Delta I + \text{const}$

The data for critical illumination producing threshold response to flicker in the sun fish *Lepomis* show for the rods $\sigma_{I_2} = KI_2$ for the cones $\sigma_{I_2} = K'(I_2 + \text{const})$

The data thus indicate that in all these experiments essentially the same visual function is being examined, and that the recognition of the production of a difference in effect by alternately illuminated stripes takes place in such a way that $d(\Delta I)/d(\sigma_{I_2}) = \text{const}$, and that ΔI is directly proportional to I (or " I_2 ," depending on the nature of the experiment)

It is pointed out that the curve for each of the cases considered can be gotten equally well if mean I or σ_I is plotted as a function of the independent variable involved in the experiment

Certain consequences of these and related facts are important for the treatment of the general problem of intensity discrimination

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THE RESISTANCE OF DROSOPHILA TO ALCOHOL

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I

The interpretation of data relating frequencies or speed of activities and processes in living organisms to the temperature of the organism may be assisted by the examination of phenomena which are deliberately selected and experimentally made to be of a known kind of complexity. It has been supposed, not infrequently, that the activities of living cells and cell aggregates are inherently so complex that simple, reproducible, comprehensible relationships to magnitudes of a known, controlled relevant variable are not to be expected (*cf* Clark, 1933). A direct way to test this notion is to examine phenomena which are known to be complex (*e g*, Crozier and Stier, 1925-26). If in terms of a type of analysis thought to give simple understandable results in "simple" cases, simplicity should apparently be revealed also in cases known to be complex, the method of interpretation might well be open to suspicion. On the other hand, it is perfectly obvious from the analysis of variation of performance in organic activities (Crozier, 1929, 1935) that the assumption of "complexity" in the sense indicated is not only gratuitous, but is inadmissible.

Examples of the complex type of situation with which one may attempt to deal are found in a number of cases where experiments have been made seeking to determine the temperature coefficients for the actions of drugs. It is clear that for fruitful treatment one must be able to dissociate (1) the relation between temperature and the time course of the action of the reagent employed, in terms of the organic performance which is measured, from (2) the effect of temperature upon the organic activity itself (Crozier and Stier (unpublished data), Crozier, 1934-35).

A further instance, which also appealed to us as possibly opening

avenues for genetic experimentation, was suggested by the experiment reported in summary by Pearl, White, and Miner (1929). As a contribution to the estimation of the physiological nature of senescence they measured the times required for adult *Drosophila* of a particular strain to become "anesthetized" by the vapor of ethyl alcohol as a function of age and of sex, at 25.5°C. Their discussion of the results makes it clear that they considered the measured times to reflect the way in which the age of the organism expresses itself as determining the capacity to continue "living." A means might then be available for the physiological characterization of races in this respect. The end-point for the observations is said to have been "a definite end stage of anaesthesia," just what this was is not stated, and we have not been able to learn precisely what it was, our observations indicate that reversible anesthesia by ethyl alcohol does not provide really satisfactory end-points for judgment of a given stage of the progress of narcosis. Between ages 1 and 60 days the times recorded for the achievement of anesthesia decline in an orderly fashion, from 9.6 to 4.1 minutes. They refer to these figures as measurements of "alcohol tolerance," and speak of the data as providing "an index or measure of physiological senescence—of the rate of growing old, in short—quite distinct and apart from the phenomenon of death." In this statement of viewpoint, and in the analysis offered for these data, the distribution of the occurrences of deaths in a population, with respect to age, is somehow thought of as being dependent upon an analogous distribution of the forces determining the length of life of a single individual, in the various individuals of a population, while these are still alive and for the most part quite remote from death. There is no reason whatever for supposing that the character of the age distribution of the incidence of deaths in a population can tell anything about, or be predicted from, the nature of the distribution of the magnitudes of the possibly relevant forces in the members of this population as obtained at an instantaneous cross-section of their lives. Certainly, there is no possibility of a formal statistical connection. The time course of change in these forces may have no direct relation to their intensities at a given moment, even supposing these intensities to be measurable, and we shall have occasion to demonstrate that mere survival time in alcohol is in no sense a direct measure of vital resistance, nor does it yield continuous curves as a function of age.

To the curve obtained by plotting time for anesthesia (y) against age in days (x), Pearl, White, and Miner fit the equation

$$y = 32.17 e^{-1.524 x^{1/2}}$$

for the combined data for males and females. The fit is certainly not "good." This formula they compare, however, with the Gompertz equation for the force of mortality

$$\mu = Be^x,$$

saying that the difference in the form of the exponent is "unimportant from the standpoint of theory." The equation is also compared with du Nouy's expression for the time course of change of surface tension of blood serum—

$$y = y_0 e^{-k^{1/2} t^{1/2}}$$

and they say that it gives "the same type of curve" because membrane phenomena may be involved in anesthesia. Aside from the specific forms of these comparisons, which are in point of fact mutually inconsistent, it is clear that the time for production of anesthesia in *Drosophila* by the vapor of alcohol is implicitly held to represent the time involved in a reaction between the living substance of the fly and the alcohol, at least in so far as the *shape* of the curve is concerned. The argument from the asserted similarity to the Gompertz curve is that the shape of the curve reveals the age course of the forces making for death, or conditioning "vitality."

If this type of interpretation is correct, it should be possible to investigate the way in which the reaction between *Drosophila* protoplasm and alcohol is affected by altering the temperature. This we have endeavored to test by a suitable series of experiments. It has been suggested, for example, that the temperature coefficients for protoplasmic activities are functions of age (Bělerádek, 1926, but cf Crozier and Stier, 1926-27). It turns out that the shape of the curve relating time for effect of alcohol to age cannot possibly be related directly to the forces determining the incidence of mortality in a population. The analysis of a situation of this type requires that the nature of the constants implied in the descriptive curve be tested experimentally. This means that an additional coordinate of reference must be employed, permitting the assignment of physically

meaningful dimensions to the parameters involved. The variables concerned in the test are age of fly, sex, temperature, and vapor pressure of alcohol. It will be shown that from the data there can be obtained valid measures of the resistance of the fly to alcohol vapor, as a function of age,—but that they do not have the sort of significance suggested by Pearl. There is also secured a paradigm of procedure for the interpretation of certain types of curves connecting biological effect and temperature, and for the analysis of toxic effects.

II

At least three classes of variables are concerned in the killing of *Drosophila* by alcohol vapor, with reference to the way in which the time for death is modified when the temperature is altered. These must be separately examined and allowed for in a suitable way before one can decide whether the change in time-to-death as a function of age, with other conditions constant, reveals anything whatever as to the character of a presumed reaction between *Drosophila* protoplasm and the alcohol, or whether indeed the time-to-death is a legitimate measure of resistance to alcohol (or of tolerance). A resistance is to be measured only in terms of the relationship between the resistance and the force which it opposes, it does not have the dimensions of a time, to assume that it may be proportional thereto implies a mechanism of the effect observed, knowledge about which must be obtained by independent tests. The variables are (1) the vapor pressure of the alcohol, (2) the speed of invasion of the body of the fly by the alcohol vapor, and (3) the reaction of the substance of the fly with the penetrating alcohol.

The flies used were of a Florida strain which had been inbred by pair matings through nineteen brother x sister generations. We are indebted to Dr. M. Demerec of the Carnegie Institution for a supply of the foundation stock. They were raised under uniform conditions of culture at 25°. The flies selected for the tests were also kept at this temperature until used. The experiments under consideration required series of sexually segregated flies ranging in age from 1 to 50 days. Each morning and evening at regular times all the flies were shaken out of the breeding bottles. They were then anesthetized (ether) and the sexes segregated, the males being placed in one fresh culture bottle, the females in another. Thus we had a group of male flies not more than 12 hours old, and a similar group of females. These were kept in an incubator for future use. At

the end of 50 days we had a series of segregated flies ranging in age from 1 to 50 days. Every 7 days the aging flies were shaken into new bottles containing freshly made food. In any cases of mold or bacterial contamination or of fertilized females the bottles as well as the flies were discarded.

When placed in air containing alcohol vapor a fly becomes after a variable latent period intoxicated, it staggers or flits about jerkily. This is followed by a stage of partial narcosis during which the fly lies on its back and is more or less immobile. Many flies continue to move their legs sporadically. Sometimes, after a fly has been on its back for several minutes it will right itself and stagger about again before collapsing. Occurrences of this type introduce a large uncertainty into the use of a condition of anesthesia as an end point for judging the time of action of the vapor. The death point is much more definitely recognizable. The prone

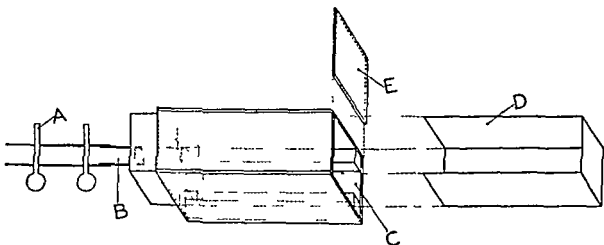


FIG 1 Chamber in which *Drosophila* were exposed to alcohol vapor. C, trough containing absorbent paper saturated with alcohol. D, basket of wire screening inserted on the shoulders at the sides of C. E, glass plate closing end of chamber. A, pinch-cocks on rubber tube, forming small air lock for admission of flies. B, glass tube in cork plug.

fly suddenly flexes its previously extended legs so that they are then interlocked. Before arriving at this stage, flies removed from the alcohol vapor will usually recover. After this movement has been exhibited, however, recovery does not occur. The drawing in of the legs is precise and sudden, and provides a definite end point.

The time elapsing between the introduction of a fly into alcohol vapor and its death was measured with a stop watch to the nearest 5 or 10 seconds. The usual procedure was to introduce a group of flies into a plate glass chamber in which the atmosphere was saturated with alcohol. The chamber (Fig 1) measured (inside dimensions) $13 \times 22 \times 60$ mm. It contained on the bottom a trough for the accommodation of strips of paper towelling which had been soaked in redistilled 'absolute' ethyl alcohol. Above the trough there was inserted a basket of brass wire screening. This effectively prevented the flies from getting

into contact with the wet towelling. The open end of the chamber was closed by a sliding glass plate, sealed with vaseline. The opposite end of the chamber carried a sealed-in cork plug. A glass tube extended through the cork into the chamber. Flies in groups of from five to fifteen were shaken through a funnel and rubber tubing into the glass tube. Two pinch-cocks on the rubber tubing produced a small intermediate chamber or air-lock. The flies were shaken from this air-lock into the alcohol chamber, after some experience this could be accomplished very quickly and neatly.

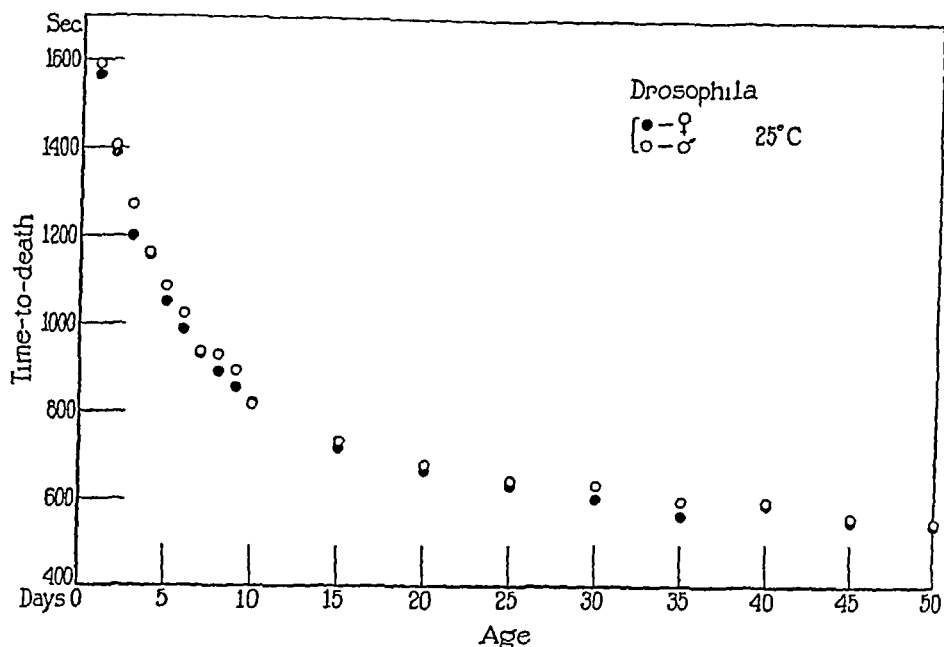


FIG 2 Mean time-to-death for ♂ and for ♀ *Drosophila* in air saturated with vapor of ethyl alcohol at 25°C (Table I) See text

Usually the chamber was placed on the stage of a dissecting microscope for determination of the times-to-death. The procedure was repeated until observations had been obtained on thirty or more flies at each age (see tables), at each of the following temperatures 10°, 15°, 20°, 25°, 30°, 35°C.

For determinations at temperatures below 25° the complete apparatus was moved into one or another of several constant temperature rooms, the temperature of which had been regulated to the desired level. The temperature was continually observed with a thermometer with the bulb as close as possible to the alcohol chamber. For determinations at 25°, 30°, and 35° the apparatus was placed in a large thermostatically controlled air bath with a plate glass top. Manipulation was possible through two apertures for the hands. The ocular

tubes of the microscope projected into the chamber through a hole in its top. The temperature fluctuations did not exceed ± 1 at the extreme.

Table I contains the results of determinations of time to death at 25°C as a function of age, for males and for females, in an atmosphere saturated with alcohol vapor. The course of the data (Fig. 2) is in a general way quite similar to that given by Pearl, White, and Miner (1929) for "anesthesia." The time to-death apparently declines in a

TABLE I

Time to-death for *Drosophila* in air saturated with vapor of ethyl alcohol, at 25°C . Each mean time is the average of about 30 measurements.

Age	$\sigma\sigma$			Time			$\sigma^{\circ}\sigma^{\circ}$		
days	sec	\pm	σ				sec	\pm	σ
1	1,566	\pm	53.6				1,590	\pm	79.6
2	1,389		79.9				1,407		58.3
3	1,200		73.0				1,272		71.0
4	1,158		69.6				1,165		80.0
5	1,052		79.2				1,088		60.5
6	989.0		67.4				1,025		53.4
7	934.0		82.7				938.3		71.4
8	893.8		68.2				932.3		68.8
9	857.7		63.5				896.0		80.0
10	822.1		60.6				818.3		63.8
15	718.4		62.2				734.6		76.1
20	666.8		62.4				681.7		60.8
25	633.0		69.8				645.0		73.6
30	604.7		70.2				635.8		74.2
35	564.6		75.9				597.3		86.7
40	590.0		86.6				596.3		78.4
45	552.1		77.5				561.2		76.8
50	542.8		83.5				549.2		78.2

rather orderly fashion with age, and is consistently longer for males than for females. We do not find that the curve can be satisfactorily fitted by an equation of the type used by Pearl. No smooth curve is drawn through these observations because, as will be shown presently, the succession of points is properly to be fitted by a line with sharp changes of curvature and is a composite affair.

To obtain from such observations a number which may be taken to

represent the resistance of the fly to alcohol requires the introduction of additional variables, in this case vapor pressure and temperature

III

To cause death it may be presumed that a certain amount of alcohol must have entered the fly. This quantity, which we may call X , might be expected to appear as a function of age of fly, sex, temperature, and vapor pressure of alcohol. When the outer concentration of alcohol is unchanged by the diffusion of alcohol into the flies, a condition assured in the present case by the arrangements under which observation is made, the rate of diffusion at death is constant at each value of P and of X . The time t for attainment of X will then be a declining rectilinear function of P , for each value of X ,—that is, for each age and sex,—on the assumption that X itself depends only on sex and on age, and is independent of P . X could appear to depend on P if a “defensive” reaction of the fly (involving its respiratory movements, for example) varied in over-all efficiency with P or t or both, but there is no indication of necessity for such a supposition. On this basis, the proportionality constant K is a coefficient of invasibility.

The essential rôle of physical diffusion of alcohol in this matter, to the practical exclusion of any effect for which the temperature might be held responsible, was tested in several ways. It was desired to compare the times-to-death at different temperatures for flies of the same age in atmospheres containing different partial pressures of alcohol. In a semi-quantitative way this was done by the following method.

A gas-collecting bottle was closed with a two-hole rubber stopper, through one hole extended a glass tube by which flies could be introduced, one at a time, through the other hole extended a tube which had been drawn out to a very fine tip. The pipette tube was partially filled with absolute alcohol, a rubber bulb closing the pipette made it possible to form at the tip successive droplets of alcohol of equal size. A known number of drops could then be released into the bottle, this was uniformly done at 24°C. During the formation of a droplet, of course, considerable evaporation of alcohol occurs, and the amount evaporated from a given droplet forming in the closed space will be increased by an amount which declines in a nearly exponential fashion with the number of droplets formed precedingly. Consequently, the vapor pressure of alcohol cannot be taken from the number of droplets released in the closed bottle, but in successive trials, atmospheres containing graded amounts of alcohol can be duplicated.

Table II contains the results of measurements made in this manner. It is perfectly clear that although the resistance of the flies does depend upon their age, and upon the vapor pressure of the alcohol, it is not distinguishably dependent upon the temperature, the small effect of a temperature coefficient of diffusion is masked by the inevitable

TABLE II

Time to-death for *Drosophila* ($\sigma^7\sigma^7$ and $\eta^7\eta^7$, mixed) of two ages, with the P E's ($n = 10$), in atmospheres containing different concentrations of alcohol at each of two temperatures. See text, the concentrations are given arbitrarily in numbers of drops of alcohol introduced into a fixed volume of air under controlled conditions, the "number of drops" is not directly proportional to the vapor pressure of alcohol

Age 1 to 6 hours		
Drops alcohol	$T = 15$	$T = 30$
	min.	min
10	90.0 \pm 1.07	93.9 \pm 1.44
15	52.8 \pm 1.58	54.6 \pm 1.23
20	35.6 \pm 0.677	37.2 \pm 0.733
30		28.9 \pm 0.592
40		21.9 \pm 0.592
50 (saturation)		20.8 \pm 0.621
Age 10 days		
Drops alcohol	$T = 15$	$T = 30$
10	57.2 \pm 0.902	54.6 \pm 1.13
15	35.2 \pm 0.733	38.8 \pm 0.818
20	24.1 \pm 0.648	22.3 \pm 0.762
30		13.6 \pm 0.779
40		15.0 \pm 0.733
50		12.4 \pm 0.451

variations in resistance from one individual to another. The data of Table II are plotted in Fig. 3.

To test the correctness of this view, experiments were performed in which the time to-death was obtained in the case of flies exposed at different temperatures to the vapor of ethyl alcohol saturated at 10°C. A vessel at 10°, containing an atmosphere saturated with alcohol vapor at 10°, was connected by a capillary tube with a chamber held at 10°, 20°, 25°, or 35°. It was assumed that, after diffusion had proceeded

for some time, the chamber at the higher temperature would contain alcohol vapor in equilibrium with liquid alcohol at 10° . The two chambers were of the same size. The data from this experiment are summarized in Table III. They show that in the case of flies 1 to 6 hours after hatching there is a slight but perhaps significant fall in the

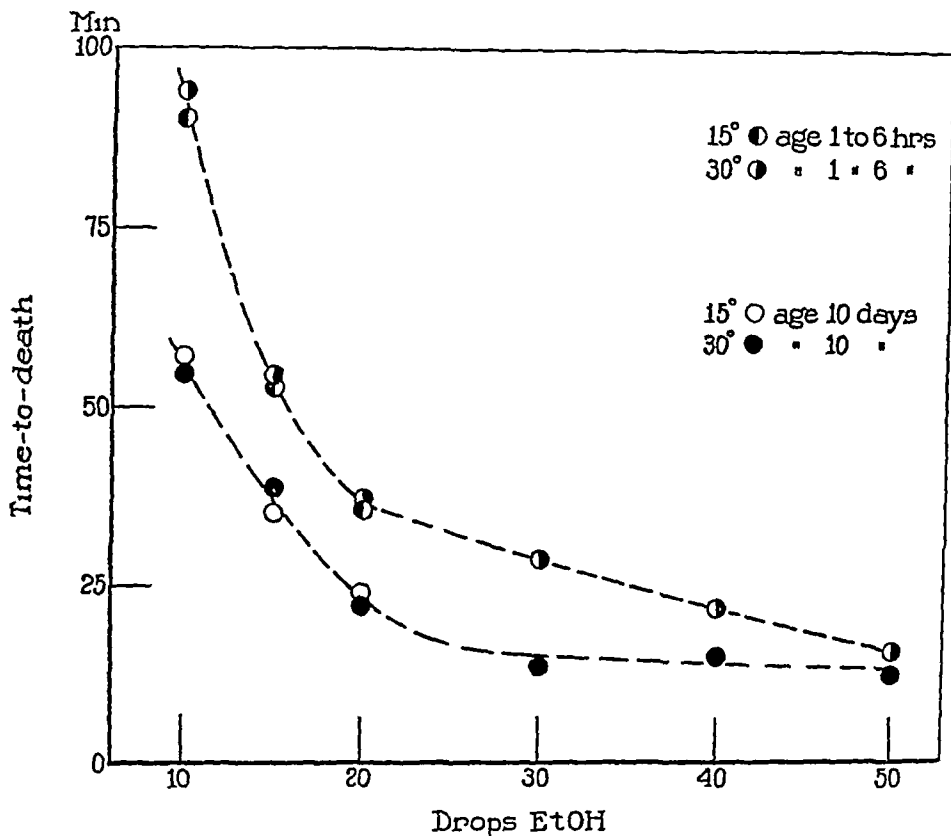


FIG 3 Vapor pressure of alcohol (arbitrary scale of unequal units based upon "number of droplets of alcohol," see text), rather than temperature, is the significant variable determining time-to-death in flies of a given age (Table II), both sexes

time-to-death from 10° to 25° , with a slight, possibly significant, rise again at 35° , variation in the proportion of males to females in the sample could be responsible for the differences, or variations in age. With flies 15 days old, there is no difference in time-to-death at 10° and at 35° , with vapor pressure of alcohol the same. This is consistent with the conclusion already drawn that the change in time-to-death

is in by far the greater part determined by the vapor pressure of the alcohol in the atmosphere. To only a very slight extent, if detectably, does it seem to be determined by the effect of temperature upon the invasion of the fly's body by the alcohol, or by any reaction of the fly connected with or influencing its respiratory movements.

We assume, then, from the foregoing tests, that the rate of invasion of the body of *Drosophila* by alcohol is the variable which determines the time of death in these experiments. When dealing with saturated atmospheres, and obtaining different partial pressures of alcohol by varying the temperature, we may as a first approach consider only the differences in vapor pressure of alcohol. We expect that in general, with flies of one sex and age, the time-to-death will be a rectilinear function of the vapor pressure of alcohol, although at the highest

TABLE III

Drosophila ($\sigma\sigma$ and $\varphi\varphi$) exposed at different temperatures to air saturated with alcohol vapor at 10°C . Mean times to-death, in minutes, with P.E. = $n = 10$

Age of flies	Temperature $^\circ\text{C}$			
	10	20	25	35
1 to 6 hrs	37.3 ± 0.648	35.4 ± 0.621	33.5 ± 0.648	36.8 ± 0.564
15 days	17.0 ± 0.677			18.1 ± 0.536

temperatures (and partial pressures) some deviations from this would not be surprising. The vapor pressure of ethyl alcohol has a rather high temperature coefficient, the vapor pressure (P) in equilibrium with liquid alcohol at absolute temperature T is described by the Clausius-Clapyeron equation

$$dP/dT = -(L/RT^2)$$

where L , the latent heat of vaporization, is 11,300 cal per mol (Fig 4). For any one age of fly, on the diffusion hypothesis, we may then translate the temperatures used into their equivalents in vapor pressure of alcohol, and expect to find that time-to-death (t) is a declining rectilinear function of P . This is illustrated in Fig 5 (data in Table IV), and the expectation is very satisfactorily met. It is clear that the

criterion of a death-point used is one which can be employed in a consistent, unequivocal manner

It is important to notice that the slope of the line in Fig 5 is a valid index of the resistance of the flies ($\sigma^7 \sigma^7$, 8 days) to alcohol vapor. The slope is conveniently expressed as $-\Delta t/(\Delta P=40 \text{ mm})$, and in Fig 5 it has the value $-332 \text{ sec}/40 \text{ mm}$. It is a real (though inverse) measure of *resistance* to alcohol, since, independent of P , and

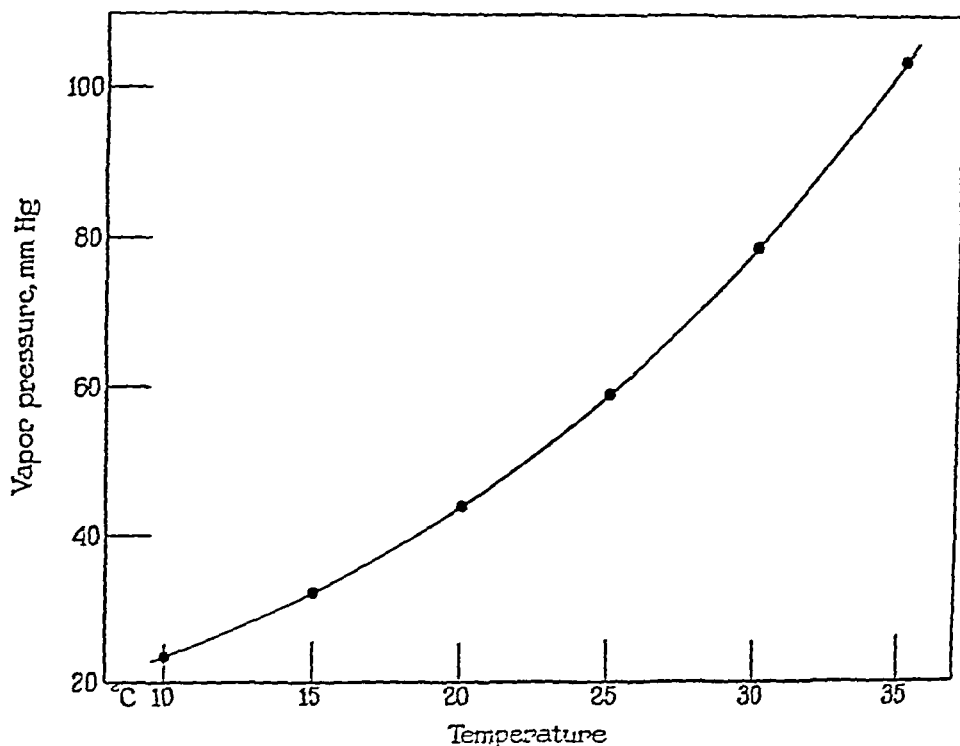


FIG 4 The vapor pressure of ethyl alcohol as a function of temperature (data from International Critical Tables), the curve is that for $dP/dT = 11,300 RT^2$

of t , it measures the decline of time of survival per unit increase in concentration of alcohol vapor. This unit of *measurability* we will call S . It is not to be confused with protoplasmic *susceptibility*, it says nothing about X , the minimum lethal dose of alcohol. The mere measure of time of survival does not give information of the same kind as S , and indeed its indications (as we shall show later) may be in this respect quite misleading.

IV

The resistance to penetration of alcohol depends in a systematic way upon age and sex. The observations of time to death are collected in Table IV. The data for females are plotted in Fig 6, for males in Fig 7. For reasons already considered the temperature is disre-

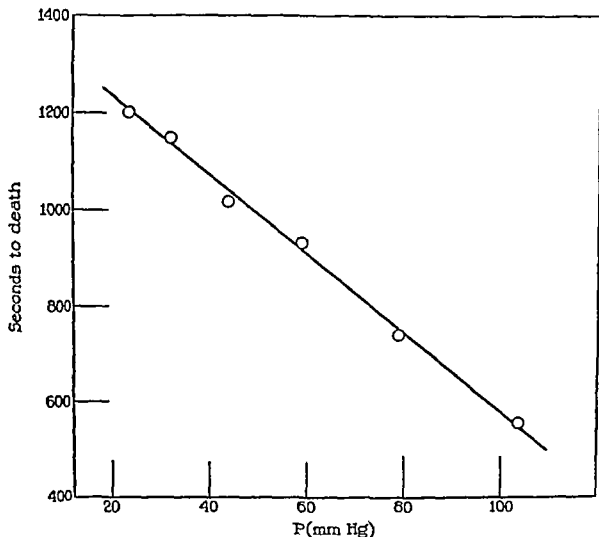


FIG 5 The time-to-death is a declining rectilinear function of vapor pressure of alcohol, independent of the temperature used to maintain the vapor pressure at a given level (see text Table IV), data for male flies 8 days old

garded, its only significance in the experiments is to regulate the vapor pressure of alcohol. At each age the time to death is a rectilinear function of the vapor pressure of alcohol. At the highest vapor pressure the flies 20 days old or older show a systematic decline in resistance to invasion of alcohol, this must be due primarily to the

TABLE IV

Time to death, sec., and σ

Mean time-to-death for males and females, at various ages (age in days \pm 6 hours), with the standard deviations of these means, at 6 vapor pressures of alcohol

Age	$P = 23.6 \text{ mm}$ $T = 10^\circ$	32.2 mm 15°	43.9 mm 20°	59.0 mm 25°	78.8 mm 30°	103.7 mm 35°
days						
1 f	2,092 7 \pm 101 4	1,934 4 \pm 71 7	1,761 7 \pm 75 7	1,565 8 \pm 53 6	1,257 0 \pm 75 1	921 0 \pm 100 1
m	2,135 5 \pm 97 1	1,980 7 \pm 66 8	1,825 3 \pm 80 2	1,589 7 \pm 79 6	1,288 5 \pm 66 6	956 1 \pm 96 5
2 f	1,896 2 \pm 86 7	1,768 2 \pm 49 8	1,579 2 \pm 77 2	1,389 4 \pm 79 9	1,115 7 \pm 74 2	803 0 \pm 88 8
m	1,892 3 \pm 85 1	1,771 9 \pm 83 2	1,586 8 \pm 89 8	1,406 9 \pm 58 3	1,157 6 \pm 76 1	840 0 \pm 83 8
3 f	1,721 7 \pm 105 5	1,576 2 \pm 79 6	1,425 5 \pm 94 0	1,200 0 \pm 73 0	999 6 \pm 74 3	768 9 \pm 76 9
m	1,770 8 \pm 95 6	1,575 0 \pm 66 4	1,455 9 \pm 78 0	1,272 1 \pm 71 0	990 7 \pm 79 0	746 6 \pm 83 0
4 f	1,535 6 \pm 75 4	1,470 0 \pm 82 9	1,334 3 \pm 73 4	1,158 3 \pm 69 6	934 1 \pm 73 0	701 0 \pm 95 5
m	1,555 5 \pm 84 0	1,448 7 \pm 46 4	1,363 2 \pm 69 7	1,164 6 \pm 80 0	957 9 \pm 71 0	665 7 \pm 80 6
5 f	1,100 0 \pm 77 1	1,339 1 \pm 65 7	1,206 5 \pm 80 7	1,051 9 \pm 79 2	890 4 \pm 82 5	630 0 \pm 89 2
m	1,403 3 \pm 58 7	1,356 9 \pm 73 6	1,258 5 \pm 81 4	1,087 6 \pm 60 5	882 1 \pm 72 8	631 6 \pm 75 4
6 f	1,264 8 \pm 56 4	1,211 6 \pm 61 9	1,104 7 \pm 80 8	989 0 \pm 67 4	805 2 \pm 78 8	589 3 \pm 80 3
m	1,287 1 \pm 78 0	1,207 7 \pm 68 8	1,144 1 \pm 66 7	1,025 4 \pm 53 4	816 6 \pm 70 2	620 7 \pm 79 7
7 f	1,232 5 \pm 73 2	1,144 0 \pm 64 9	1,051 4 \pm 86 2	934 0 \pm 82 7	777 4 \pm 68 4	593 3 \pm 85 2
m	1,242 6 \pm 74 5	1,149 1 \pm 71 2	1,078 6 \pm 58 5	938 3 \pm 71 4	777 9 \pm 66 4	564 5 \pm 76 1
8 f	1,159 4 \pm 71 8	1,100 3 \pm 72 0	994 5 \pm 72 4	893 8 \pm 68 2	724 7 \pm 66 3	516 7 \pm 81 5
m	1,203 5 \pm 88 8	1,149 4 \pm 71 0	1,017 9 \pm 84 4	932 3 \pm 68 8	741 0 \pm 89 1	559 0 \pm 87 2

9 f	1 100 0 ± 78 0	1 067 3 ± 92 3	971 7 ± 71 1	857 7 ± 63 5	715 5 ± 68 4	528 6 ± 76 1
m	1 096 7 ± 73 7	1 086 3 ± 102 1	1 013 8 ± 71 4	896 0 ± 80 0	721 8 ± 74 2	488 7 ± 82 0
10 f	1 059 7 ± 47 5	1 013 8 ± 72 3	930 0 ± 73 7	822 1 ± 60 6	603 9 ± 76 4	470 7 ± 78 8
m	1 110 6 ± 56 0	1 041 0 ± 80 7	947 0 ± 56 6	818 3 ± 63 8	635 2 ± 85 4	456 0 ± 88 6
15 f	958 2 ± 81 5	935 7 ± 85 7	802 8 ± 79 8	718 4 ± 62 2	591 3 ± 84 7	427 4 ± 85 1
m	983 6 ± 74 0	930 0 ± 92 4	811 3 ± 73 1	734 6 ± 76 1	631 4 ± 86 2	436 1 ± 72 5
20 f	850 0 ± 65 5	791 5 ± 63 9	739 7 ± 81 2	666 8 ± 62 4	571 6 ± 90 0	393 4 ± 45 5
m	855 8 ± 63 4	836 5 ± 84 8	768 6 ± 65 6	681 7 ± 60 8	618 2 ± 82 4	397 1 ± 50 9
25 f	802 7 ± 83 9	747 1 ± 65 4	699 1 ± 79 0	633 0 ± 69 8	542 4 ± 73 8	367 6 ± 36 6
m	821 7 ± 76 2	774 5 ± 75 3	705 0 ± 86 6	645 0 ± 73 6	581 1 ± 77 6	368 6 ± 30 5
30 f	791 2 ± 87 2	711 3 ± 77 1	660 3 ± 58 2	604 7 ± 70 2	499 7 ± 76 1	362 7 ± 28 3
m	757 7 ± 76 9	747 2 ± 73 7	668 7 ± 66 4	635 8 ± 74 2	553 9 ± 72 2	366 2 ± 38 7
35 f	809 2 ± 78 6	679 1 ± 81 5	639 0 ± 59 6	564 6 ± 75 9	491 0 ± 75 8	352 4 ± 43 4
m	713 6 ± 76 8	693 2 ± 86 4	667 1 ± 66 6	597 3 ± 86 7	512 7 ± 72 9	344 2 ± 44 1
40 f	678 7 ± 66 7	666 2 ± 74 7	599 7 ± 81 0	590 0 ± 86 6	487 6 ± 72 4	341 9 ± 46 2
m	713 2 ± 78 8	668 3 ± 89 1	620 0 ± 75 2	596 3 ± 78 4	506 2 ± 75 6	338 6 ± 93 5
45 f	679 1 ± 86 9	636 9 ± 89 8	609 1 ± 68 7	552 1 ± 77 5	472 7 ± 69 1	316 2 ± 39 6
m	686 3 ± 81 4	666 3 ± 91 8	634 1 ± 80 6	561 2 ± 76 8	483 2 ± 81 6	317 5 ± 29 8
50 f	653 9 ± 72 2	626 7 ± 69 4	611 9 ± 56 6	542 8 ± 83 5	469 6 ± 69 6	312 8 ± 47 3
m	676 6 ± 84 4	658 7 ± 66 4	602 8 ± 73 7	549 2 ± 78 2	480 0 ± 72 0	306 5 ± 40 6

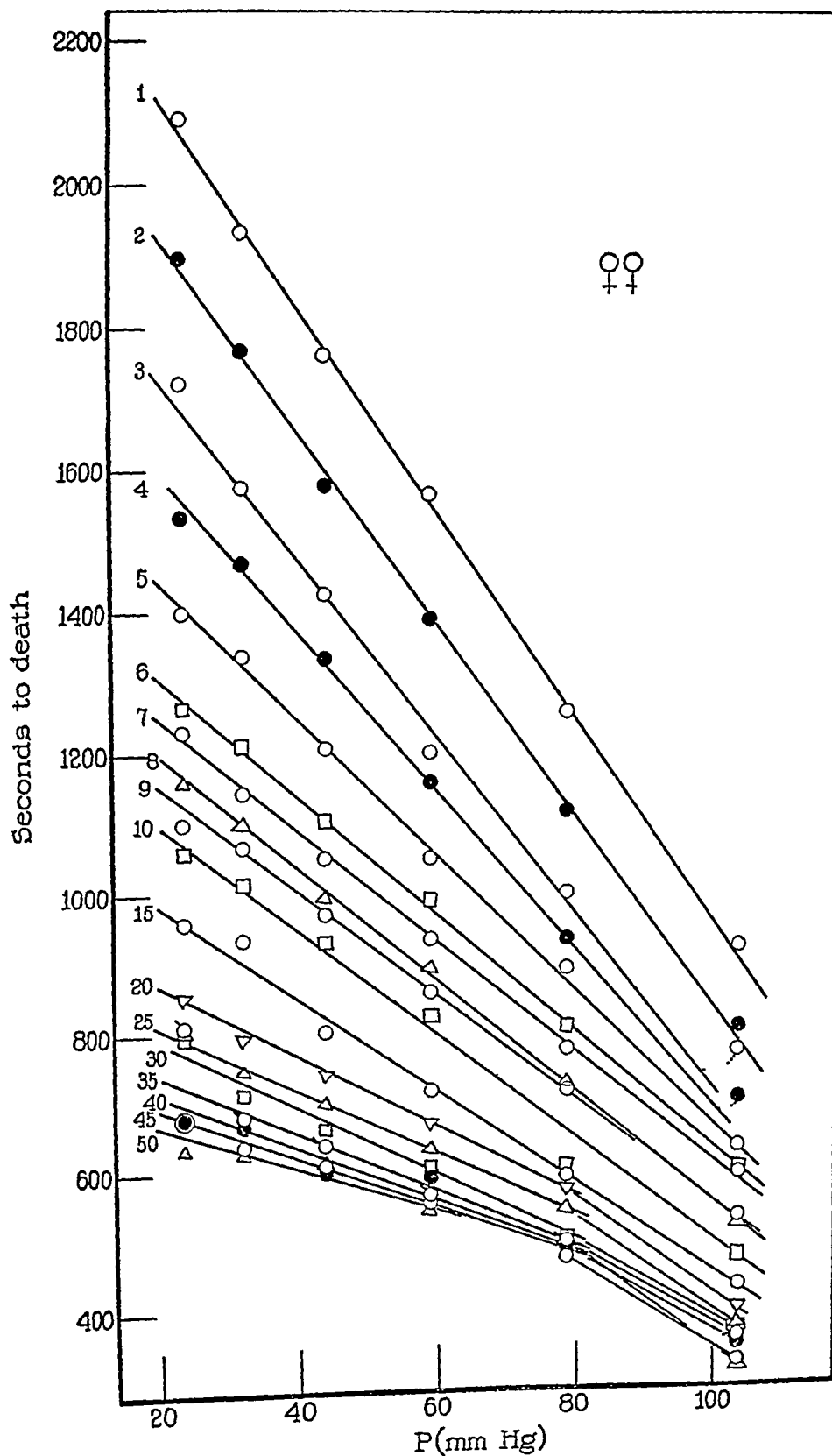


FIG 6 Time-to-death as a function of vapor pressure of alcohol for female *Drosophila* at various ages after emergence, age in days indicated on each line

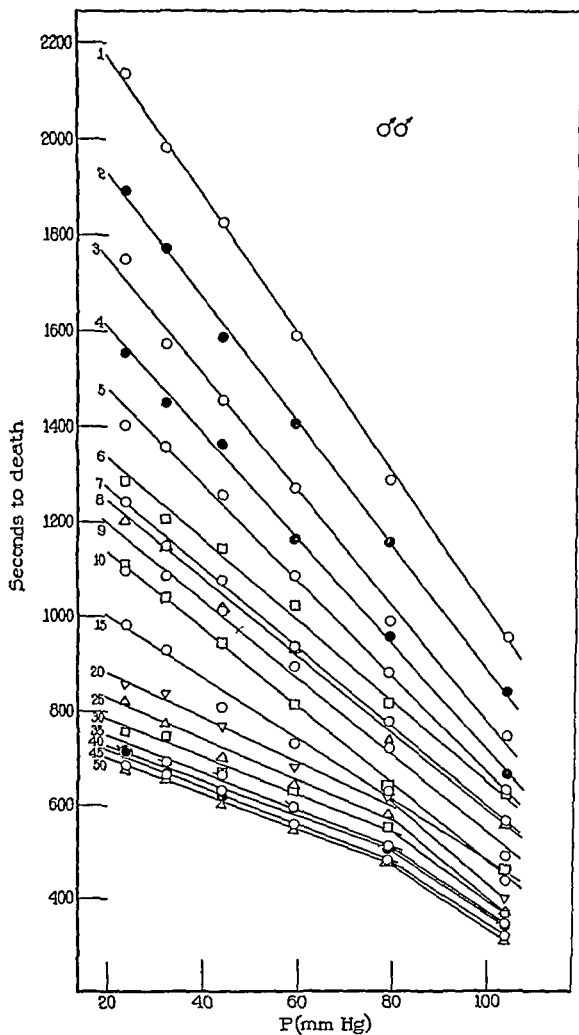


FIG 7 Time to-death as a function of vapor pressure of alcohol males at different ages

influence of the high vapor pressure of alcohol, rather than to the temperature (35°), since at 35° flies in alcohol vapor saturated at 10° die after the "10°" time, showing no certain effect of the high temperature upon the killing process (*cf* Table III)

The slope of the t, P graph decreases with advancing age. Since the ordinate position of the graph is higher the younger the animal, it is obvious that two criteria which might easily be employed for the estimation of resistance (or "tolerance") as a function of age give results which are in sharp contrast. The invasion of the fly by alcohol is easier the younger the imago, but it is clear that the quantity of alcohol required to kill is greater the younger the fly. The low temperature coefficient and the character of the t, P graph alike and independently show that the relation of t and P is determined by diffusion of alcohol into the fly, it cannot be supposed that the times observed at one value of P are capable of measuring the comparative "vital resistance" as a function of age, since the lethal quantity of alcohol (X) must be supposed to be determined only by age and by sex of fly, and not by P or by the temperature,—otherwise, the graphs in Figs 6 and 7 could not be rectilinear.

The relationship between age and lethal dosage here observed is somewhat unusual. In silkworms, for example, given arsenic by mouth, the increment in $1/t$ per unit increase of dose is greater the younger the instar, while the minimum lethal dose *increases* with advancing age (Campbell, 1925-26).

It might be suggested that if one were to employ the view exploited by Pearl, White, and Miner (1929) a mortality curve could perhaps be obtained by extrapolation on the ordinate axis at $P = 0$. But this would be quite incorrect, for several reasons—chiefly because the observed times-to-death are "times-to-death in the presence of enough alcohol to kill," and extrapolation to lower partial pressures of alcohol than the minimum lethal could not give figures with dimensional significance.

Nor can it be supposed that the manner of change of resistance to alcohol as age advances could be obtained in another way, namely by finding from Figs 6 and 7 the vapor pressure of alcohol required to kill at a constant time. Disregarding complications due to the fact that at constant P the shape of the curve for "killing" would probably depend

on the criterion of death employed (*cf* Rahn and Barnes, 1932-33, Oster and Arnold, 1934-35), it is also true that the shape of the curve for P_t vs age, where P_t is the vapor pressure of alcohol required to kill in t seconds, depends upon the time chosen, and is not smooth. These curves might have a simple significance if the various P_t graphs (Figs 6, 7) did in fact converge upon one focus on the t axis, but they do not.

TABLE V

The slope of the curve relating time to-death to vapor pressure of alcohol as a function of age the slope is $S = -\Delta t/(\Delta P = 40 \text{ mm})$

Age	S	
	t	m
days		
1	586	586
2	546	521
3	502	492
4	456	446
5	388	405
6	341	344
7	321	340
8	320	332
9	300	331
10	300	328
15	202	261
20	193	191
25	192	175
30	180	160
35	144	147
40	143	146
45	138	157
50	124	149

The slope constant S , computed from $S = -\Delta t/(\Delta P = 40 \text{ mm})$, is shown as a function of age in Table V and in Fig 8. It is apparent that a continuous smooth curve cannot be used to describe these coefficients of invasibility. The reciprocals of S , $1000/S = R$, have the dimensions of change in partial pressure of alcohol required to produce a fixed alteration in time to-death, and thus in the time required for the diffusion of a quantity of alcohol which is fatal (Fig 9).

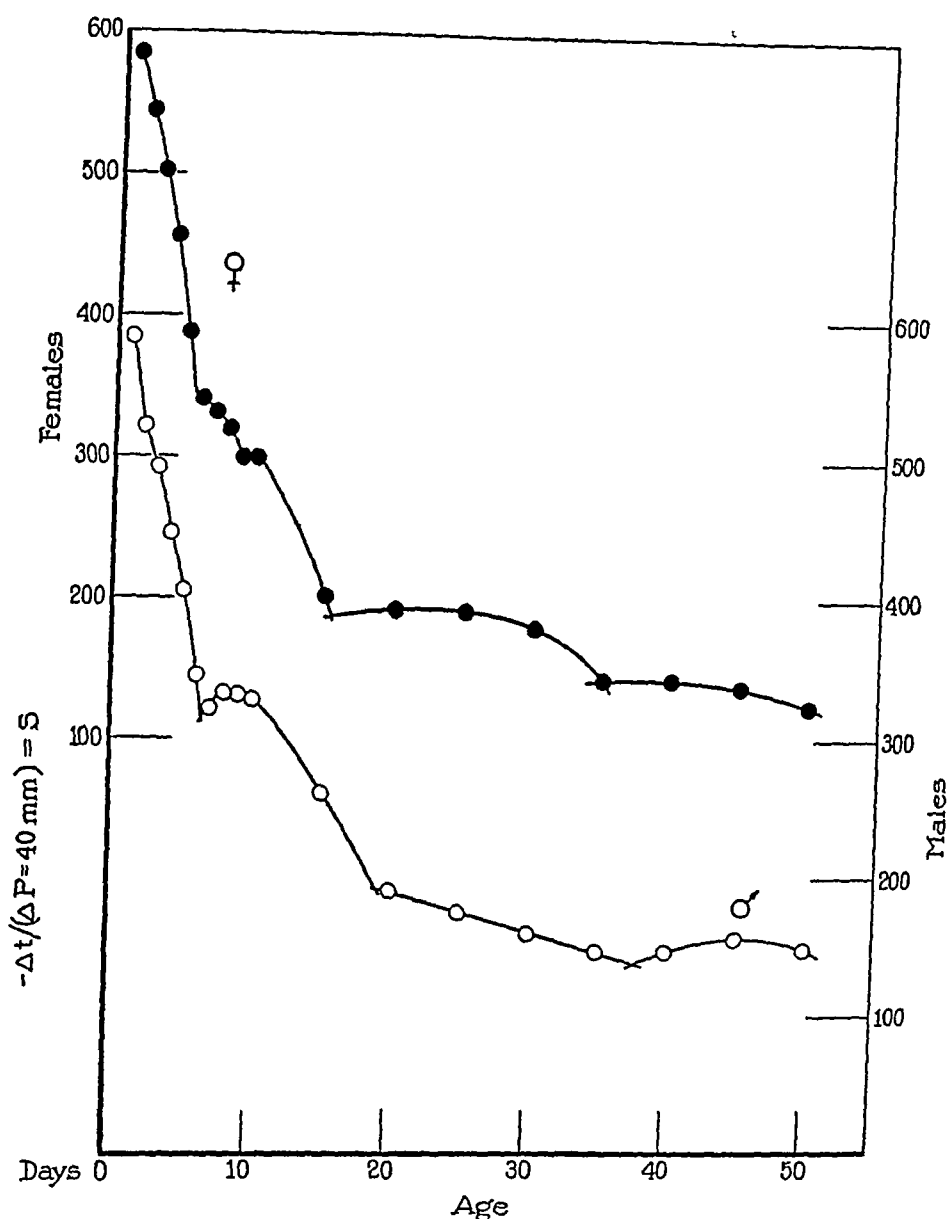


FIG 8 The slopes of the lines in Fig 6, $S = -\Delta t / (\Delta P = 40 \text{ mm})$, which are measures of invasibility by alcohol, decline in a particular fashion for males and for females

This latter curve also shows, of course, that the resistance of the fly to penetration by alcohol from its vapor increases as a function of age and exhibits successive cyclic variations. These fluctuations in relative

invasibility differ significantly in the two sexes, they are not entirely synchronous, although the general character and extent of the changes are similar in males and in females.

We can obtain independent evidence regarding cyclic fluctuations in resistance by extrapolating in the opposite direction. By extension of the lines in Figs. 6 and 7 to $t = 0$ we obtain estimates of the ideal vapor pressure required to kill instantaneously at each age. In this case the factor of invasibility is eliminated. In Table VI these values of P_0 are given, and they are plotted in Fig. 10. P_0 at ages above 20

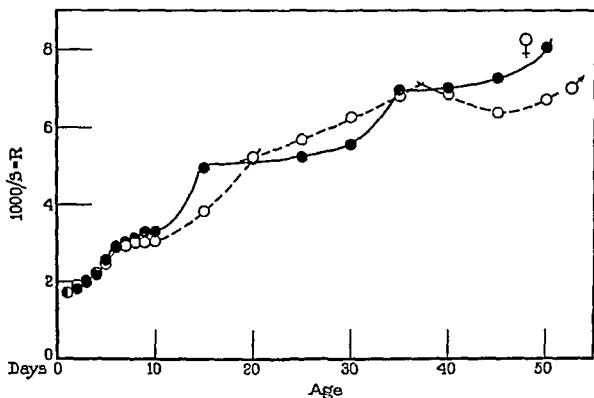


FIG. 9. The reciprocals of S (Fig. 8) measuring the resistance of *Drosophila* to invasion by vapor of ethyl alcohol as a function of age. $R = 1000/S$.

days is estimated from the final slopes at the two highest values of P . It is apparent that the resistance is not a simple function of age, but goes through several maxima. It is also apparent that neither from the standpoint of invasibility (Figs. 8, 9) nor of resistance to the toxic influence of the alcohol (Fig. 10) can it be said that the males or the females are systematically the more resistant, despite the in general distinctly longer survival times of the males at any given vapor pressure of alcohol (Table IV).

If X , the internal concentration or amount of alcohol which is just fatal, changes with age and depends on the sex, then the time taken to produce X at constant P should be inversely proportional to S , the coefficient of invasibility, and proportional to X , since dx/dt is constant

$$t_1 = KX_1/S,$$

where X_1 is the vapor pressure of the internal alcohol required for death after time t_1 . $-K$ should then be directly proportional to P ,

TABLE VI

From Figs 6 and 7 there are obtained values of P at zero survival time, by extrapolation to the P axis. See text

Age	P_0, mm	
	f	m
<i>days</i>		
1	165 0	169 6
2	161 0	168 3
3	156 5	163 9
4	160 8	164 7
5	168 9	167 0
6	173 8	176 7
7	172 0	171 0
8	168 6	171 3
9	173 0	165 3
10	166 0	158 1
15	168 5	173 2
20	160 0	151 5
25	160 3	148 5
30	168 0	153 5
35	167 9	154 0
40	160 8	153 0
45	153 0	150 8
50	149 0	148 0

for each age and sex. It is apparent that since S is a constant for each age this will be formally correct, and will result in graphs exactly analogous to those in Figs 6 and 7, the ordinate scale only being changed, as $KX_1 = St_1$. This means that X_1 varies with age in precisely the same way as S , and the curve of time-to-death at the minimum pressure of alcohol vapor which is lethal under the condi-

tions of these experiments must also exhibit the discontinuities shown in Fig 11. From determination of the minimum lethal pressure for flies of each sex at one age it is possible to obtain by these considerations the values of X_1 at each age, and then from Figs 6 and 7 to estimate the corresponding values of t_1 . This has been done for flies 10 days old, X_1 was estimated to be $P = 10$ mm for males

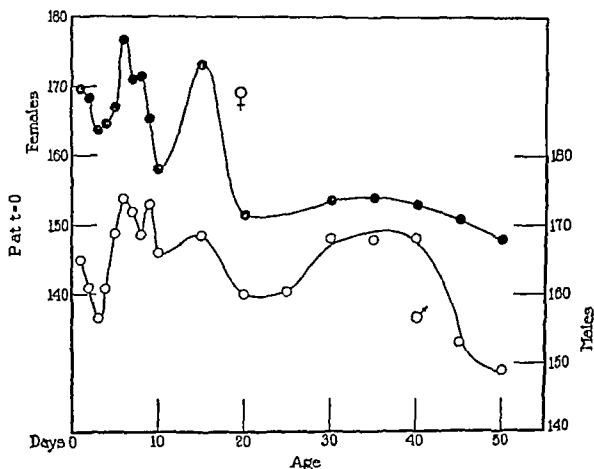


FIG 10 The extrapolation of the lines in Figs 6 7 to $t = 0$, given as P_0 in Table VI, provides estimates of the pressure required to kill *Drosophila* instantaneously. See text

The resulting curve of t_1 as a function of age is of the general character seen in Fig 11, and is accordingly not reproduced

It may be remarked that on the view that the vital resistance of the flies may be estimated in this manner under the foreshortening in fluence upon life duration of a markedly adverse condition, one should find a certain parallelism between the curves in Fig 10 and the curve of average expectation of life as a function of age. In this sense we

agree with Teissier (1934 *a*) in regarding the estimates of expectation of survival (E_x) as suitable indices of viability. Such statistics are usually exhibited as calculated after a smoothing process has been applied to the raw data upon survival times. We shall have occasion to point out that in the case of *Drosophila* such a smoothing of the observations may be quite illegitimate and may lead to the suppression of information of considerable biological interest. The E_x curve for pure lines of *Drosophila*, from smoothed data, is a continuous declining curve. Teissier (1934 *a*) has given one E_x curve, however, computed from Gowen's data on triploids (referred to by Teissier as "inter-sexes") (Gowen, 1930-31), which shows cyclic fluctuations superimposed upon the downward trend. We shall shortly have occasion

TABLE VII

At the ages where resistance (P_0) goes through a maximum, R has a sharp change in curvature, a critical point (R_c)

♀ ♀			♂ ♂	
R_c		$P_{0\max}$	R_c	$P_{0\max}$
Age, days	35	35	37	36
	15	15	20	18
	9	8	9	9
	6	6	6	6

to see that the phenomenon is apparently a general one for inbred *Drosophila*.

Before leaving the curves in Fig. 10 for the present, we note that P_0 goes through a maximum at each age where the curve of $1/S$ exhibits a sharp change of curvature, and only at these ages, for each sex (Table VII). The correspondence cannot be accidental. It signifies that fluctuations in the invasibility of the fly for alcohol, determined by age, are correlated in a specific manner with changes in the rate of change of resistance to the inner toxic action of the alcohol. The first derivatives of P_0 and of S with respect to age show completely parallel behavior. Both must therefore be determined by the general course of more fundamental changes in the fly, of which the expression in various ways may be taken to give estimates of the progress of aging.

The existence of the complexities here revealed makes it plain why the data upon time to-death as a function of age, under constant conditions of alcohol vapor pressure, cannot be fitted by one smooth curve (e.g., in Fig 2). By means of the graphs in Figs 6 and 7 estimates may be obtained of the mean time to death at each age and at constant alcohol vapor pressure, weighted by all the observations at each age. In Fig 11 such results are illustrated for $\sigma^7\sigma^7$ flies at

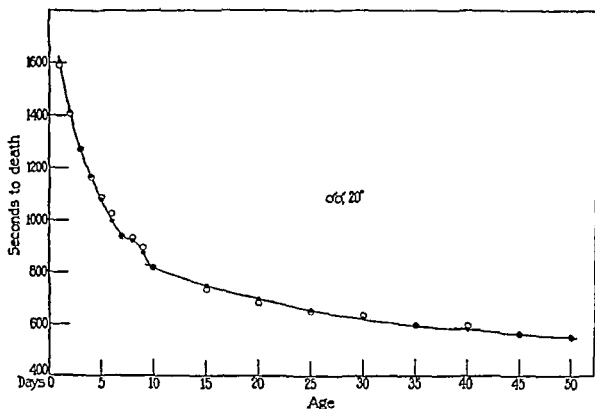


FIG 11 The solid circlets are durations of time-to-death as a function of age, interpolated on the graphs of Fig 7 at vapor pressure 25°C . The open circlets are the actual determinations. The solid circlets signify *calculated* times to-death. The discontinuities forbid passing a single smooth curve through the determinations.

20°C (cf Fig 2). The discontinuities there shown are faithfully reflected in the observations at this temperature (Tables I, IV). They result from the fact that the toxicity of alcohol and the ability of alcohol to penetrate the body of the fly are a complex function of age. Similar curves are obtained at other values of P . (The discontinuities need not appear at precisely the ages corresponding to singular points in Figs 9 and 10.)

V

In connection with problems of the general type which here concern us it is customary to tabulate measures of the dispersions of the averages of observations. It cannot be said, however, that maximum use has always been made of such statistics, even in their bearing upon the question of curve fitting. When really homogeneous data are available, observed variation in the expression of an organic property may be fruitfully related to magnitudes of a known relevant variable, in this way the capacity of the organic system to exhibit variation of performance in the matter considered may be expressed quantitatively (cf Crozier and Pincus, 1931-32, etc., Crozier, 1929, 1935).

Three large categories of variableness contribute to the differences in time-to-death in the present experiments, under fixed conditions fluctuation in the performance of the observer and of the apparatus, variations in the resistance of the flies to the toxic action of alcohol, and variations in the resistance of the flies to the penetration of alcohol.

When the standard deviations of the observations show a specific relationship to the values of an independent variable, the observer being the same, and if the conditions of observation are not materially influenced by the independent variable, one is safe in assuming that the variation indices are not a function of the process of observation,—or rather that their coefficient in terms of the independent variable is not such a function. In the present case the length of the time-to-death is the only variable directly connected with the process of observation. We assume that the flies have been correctly assorted as to sex, and that the variations connected with the introduction of the flies into the alcohol atmosphere are not significant. The maximum possible error in age of any one fly is ± 6 hours, variation introduced from this source would be largest in effect at the lowest ages, the fact is, however, that σ_i/t is lowest at these ages, hence variation from this cause is without recognizable effect. Since it turns out that σ_i is not a continuous function of t , there is no reason to suppose that t has, of itself and as connected with the process of observation, any direct influence upon σ_i . The times involved are never so short that the observer is hurried, and there is every evidence that the timing of one death does not influence the recorded time of the next observed death.

Careful examination of the properties of σ_t shows that the coefficient of variation, σ_t/t , as a function of age, exhibits the cusped character of the plot of R as a function of age. We have pointed out that R has the meaning of a *resistance*, it signifies the reciprocal of the change in time to death for a fixed increment in P , or, since the relationship is rectilinear, the change in P required to bring about at each age a given change in time to death. The relation between P and t shows that diffusion of alcohol into the fly is the significant process, the resistance to this diffusion must operate during the whole time up to the estab

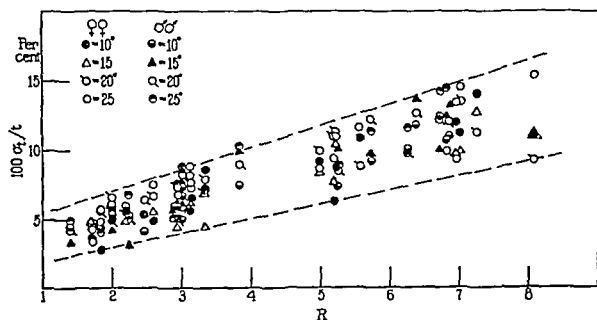


FIG. 12 Relative variation of time-to-death as a function of resistance to diffusion of alcohol. Data for individuals over 20 days old are not included at the two highest vapor pressures used (see Figs 13, 14)

lishment of the lethal condition in the fly, hence the relative variation of t may be expected to be directly proportional to R . Fig 12 shows that for $\sigma^1\sigma^1$ and for $\varnothing\varnothing$, indistinguishably, this is indeed the case. The observations at each value of P are plotted separately. At the two higher values of P , established at 30° and at 35° respectively, for flies over 20 days old, the value of R changes (Figs 6, 7, etc). These data are omitted from Fig 12, in Figs 13 and 14 it is demonstrated that for them the same kind of relationship obtains between σ_t/t and R , but that the proportionality factor is different, and also the behavior of the standard deviation of σ_t . The latter quantity is responsible for

the progressively wider scatter of σ_i/t at the higher values, when many tests of equal weights are available

These considerations provide a rational statement of the capacity of this stock of *Drosophila* to exhibit variation in time-to-death due to

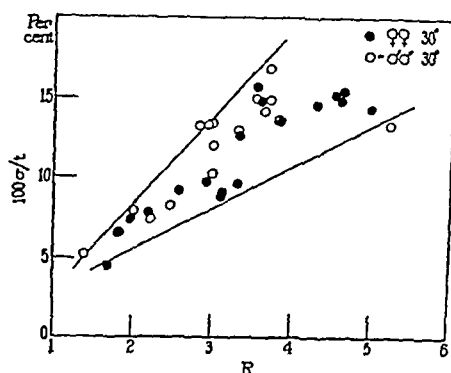


FIG 13 Relative variation of time-to-death as a function of R at vapor pressure "30°C," individuals above 20 days old

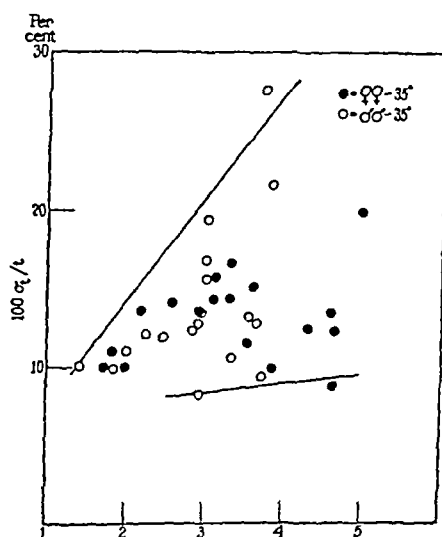


FIG 14 Relative variation of time-to-death as a function of R at vapor pressure "35°C," for individuals above 20 days old

alcohol vapor The formulation adds to the evidence that the diffusive invasion of the fly by alcohol is the only significant variable involved in the production of the lethal condition, aside from the minimum lethal internal dose, and that both are functions of age The

specific differences between males and females do not concern the variation in time to death, but have to do only with differences in R and in lethal dose. Again there is no evidence, from the variation data, that protoplasmic reaction with the alcohol determines the shape of the P, t , curve. Conceivably, different stocks, or the effects

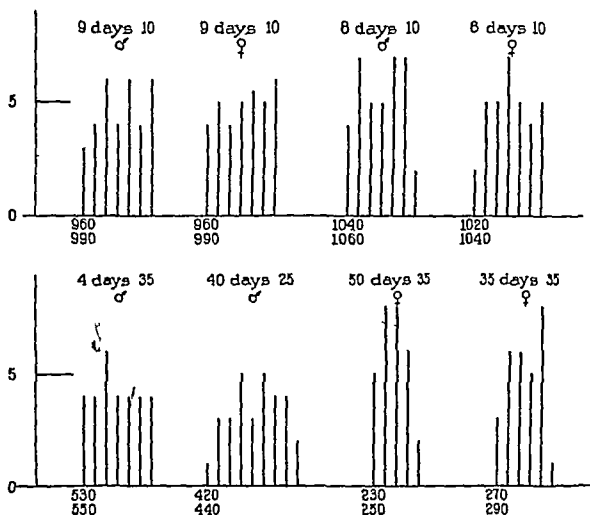


FIG 15 Frequency distribution of time to-death in groups of *Drosophila*. See text

of different nutritive conditions, could be compared on this basis of the capacity to show variation in the time to-death.

It has commonly been assumed that the susceptibilities of individuals in a homogeneous population are (or should be) distributed on a normal probability curve (*cf* Clark, 1933). In the present material this is very far from being the case. The actual distributions are characteristically of the sort shown in Fig 15 for 4 day old ♂♂ at 35°—flat distributions of a rather unusual type, they could result

from one of several causes into which we will not now inquire¹ With older flies at the highest vapor pressure of alcohol the distribution of time-to-death is of the kind shown in Fig 15 for 50 day old flies at "35°," again indicating that for the older flies, with high vapor pressure of alcohol, the variation of performance is of a different character Presumably this is determined by physical breakdown of resisting surfaces under these conditions, these latter distributions show definite skewing toward the lower times-for-death

VI

The periodic changes in resistance of *Drosophila* to penetration of alcohol from its vapor, and in the ideal vapor pressure required to kill instantly, call for interpretation The explanation suggested is that in the life of the imago there occur periodic surges of metabolic activity similar to those associated with ecdysis Prothetely indicates that moults can be experimentally suppressed There is no real objection to assuming that in imaginal dipterons there may occur "moults" which fail to materialize One expression of such processes would be assumed to be an alteration of the rate of "hardening" of the outer surface of the animal, including that of its tracheae and air sacs The linings of air sacs and tracheae of house fly and honey bee apparently contain no chitin (van Wisselingh, 1924, Campbell, 1929), but in any case there is no reason to believe that the increasing toughness of the insect exoskeleton with advancing age is due to increasing deposition of chitin The general increase in toughness with age is a well recognized property of the exoskeleton, but is due to the peripheral deposition of substances other than chitin (Campbell, 1929)

Periodic changes of the character suggested would be expected to affect diverse aspects of metabolic expression The careful scrutiny

¹ For example, it might well be supposed that at any one age there is a frequency distribution of penetrability with respect to alcohol vapor which is monomodal and essentially symmetrical, whereas the distribution of resistance in terms of toxicity of alcohol is heavily skewed in the sense that the mode is toward the low resistance side There is much general evidence from other cases to support this notion The variation in time-to-death would then be a product function of these two possibly independent variations, this could easily produce a very flat distribution

of existing data upon *Drosophila* should reveal substantial indications of their reality, if they occur. The life duration tables for *Drosophila* are usually given after the observations on survival time and age have been smoothed. The most direct approach to these figures is to consider the raw death rates (d'_x) at each age. In Fig 16 we have plotted the summated observed death rates ($\Sigma d'_x$) given for Pearl's Line 107 (Pearl and Parker, 1924, Pearl, Parker, and Gonzales, 1923). It is apparent that there occur systematic deviations in the rate of

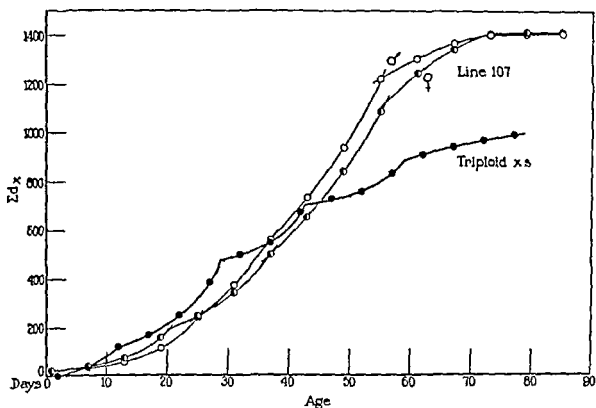


FIG 16 Summated observed occurrences of death in male and female *Drosophila* of Line 107 (Pearl and Parker, 1924) and for triploid individuals of Gowen (1930-31)

accumulation of deaths, which are in the same sense in the two sexes. This might possibly be the result of some element of the observational procedure, but the data on Vestigial show precisely the same sort of thing. A good test seems to be given by Gowen's (1930-31) tables, from his survivorship data, in which the type females and males show the effect already noted for Line 107 and Vestigial, plots are given in Fig 16 and Fig 17 of $\Sigma d'_x$ for his triploid females (3 X, 9 autosomes) and sex intergrades (2 X, 1 Y, 9 autosomes). We should expect an

accentuation of the irregularity of the $\Sigma d'_x$ curve in the triploid females, and in the group of unbalanced sex intergrades a statistical suppression of this effect, since the expression of intersexuality is variable. The curves show this to be the case, and appear to rule out the consideration of the discontinuities of rate of appearance of deaths as artifacts. When $\sigma^7\sigma^7$ and $\varphi^7\varphi^7$ data for a given stock are lumped, the discontinuities practically vanish. It is quite desirable that an analysis of such findings should be based upon daily observed death rates, but it

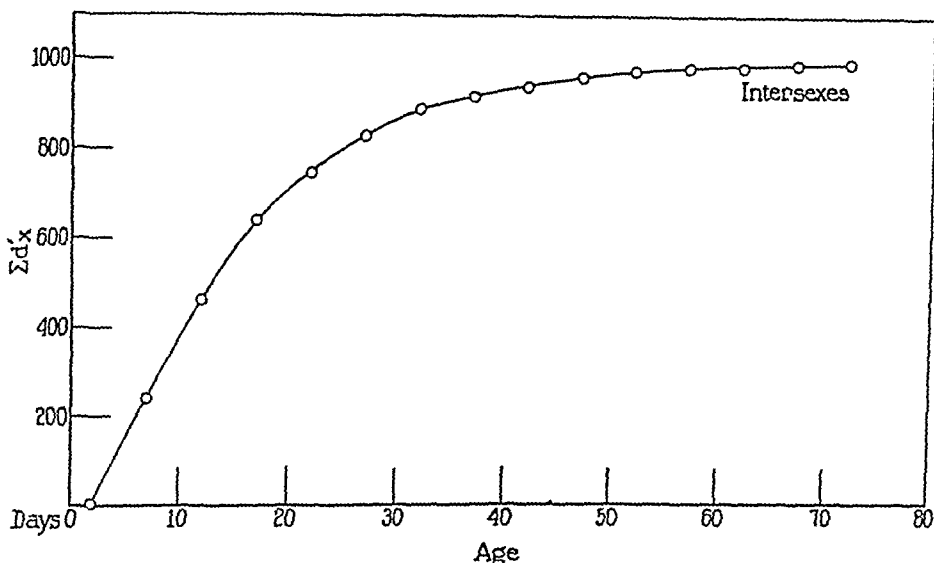


FIG 17 Summated observed occurrences of death in unbalanced intersexed *Drosophila*, data of Gowen (1930-31)

is notable that in the several sets of observations available the locations of discontinuities in the first derivative of the $\Sigma d'_x$ line are specific.

The assumption basic to the customary treatment of survivorship curves and related functions implies that an individual death is the accidental outcome of a succession of accidents, and that the derivatives of the survival ratio are not discontinuous. Since in genetically homogeneous material the form of the survivorship curves is subject to inheritance, and is determined by the genetic constitution, under fixed conditions (Pearl and Parker, 1922, Pearl, Parker, and Gonzales, 1923, Gowen, 1930-31), the initial basis for such treatment seems not to exist. If the shape of the survivorship curve is a function of genetic

constitution, then with a genetically uniform population it is not permissible to smooth the data by a least squares procedure, with a sufficiently heterogeneous population the situation is quite different. It is apparent that any derived measures of mortality for *Drosophila*

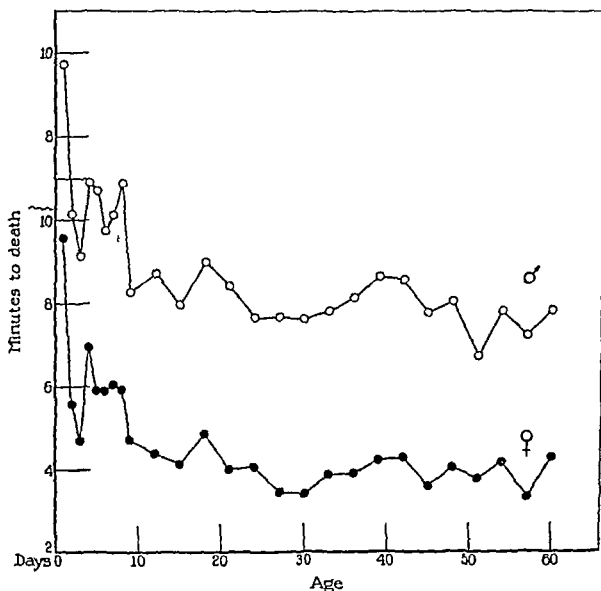


FIG. 18 Data from Pearl, White, and Miner (1929) for time to anesthesia in male and in female *Drosophila* of their Line 107. The discontinuities in change of curvature are discussed in the text.

must contain the irregularities exhibited in Figs. 16 and 17, consequently the interpretation of the "force of mortality" and related statistics, in this connection, must be recast, the outcome of experimental investigation of these matters must remain rather indefinite so

long as conclusions depend upon the comparison of artificially smoothed curves. It is suggested that in fact the likelihood of the occurrence of death is relatively greater, for *Drosophila*, at periods corresponding to the events which we have referred to as "suppressed moults," these are supposed to have the character of critical metabolic periods. In dealing with larval insects it is a commonplace that deaths accumulate at periods of ecdysis.

We do not possess life table data for the strain of flies used in our experiments with alcohol, but we are led to expect that under the same conditions of culture such a table would reveal cusps at or a little after those ages which correspond to cusps in our curve of resistance to penetration of alcohol from its vapor (Figs 6, 7), and to valleys in the curve of P_0 as a function of age (Fig 10). Additional information may be obtained by a consideration of the lipoid content of *Drosophila* as a function of age, which we shall discuss subsequently. It may be possible to secure in this way a mechanism to account for the fact that the lethal internal amount of alcohol and the invasibility of the fly vary together.

In the case of Pearl's Line 107, however, a partial comparison can be instituted. Pearl, White, and Miner (1929) measured the time for anesthesia as a function of age, with one pressure of alcohol, in this line. The comparison is unsatisfactory, because we need to know the relation between time for anesthesia and vapor pressure of alcohol, at each age. But it is apparent that the data (Fig 18) do indeed show consistent fluctuations in resistance to anesthesia, which are apparently valid and not unlike those fluctuations in the rate of incidence of dying which Fig 16 reveals, and of a type entirely similar to what we have seen in Figs 9 and 10. We are quite unable to accept the idea that a curve of the Gompertz type for force of mortality can be reasonably used to describe such observations, either theoretically (Pearl, White, and Miner, 1929, Teissier, 1934 *b*) or on any basis of expediency.

SUMMARY

The mean time-to-death (t) of imaginal *Drosophila* of an inbred line in alcohol vapor of constant partial pressure (P) is a declining rectilinear function of P for each age. The time-to-death depends upon the diffusion into the fly of an amount of alcohol sufficient to kill. It

does not depend upon any measurable property of a reaction between the substance of the fly and the alcohol which produces death. The relation between t and P is independent of temperature, but the invasion coefficient $S = -\Delta t/\Delta P$ declines with age and differs for the two sexes. The first derivative of S with respect to age exhibits sharp discontinuities. The internal alcohol required to kill declines with age, varying with S . The relative variation of t , σ_t/t , is directly proportional to the resistance to diffusive penetration of alcohol R , where $R = 1/S$.

The vapor pressure of alcohol estimated to kill instantaneously shows periodic fluctuations with age, these are precisely correlated with changes in the slope of S as a function of age.

Periodic fluctuations of invasibility by alcohol, and of the lethal dose, are interpreted as due to the incidence of suppressed moults. It is shown that in the accumulation of deaths as a function of time (age) in a genetically uniform population of *Drosophila* of one sex, similar fluctuations are apparent in the rate. The statistical smoothing of such data is not legitimate.

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SULFHYDRYL AND DISULFIDE GROUPS OF PROTEINS

IV SULFHYDRYL GROUPS OF THE PROTEINS OF MUSCLE

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(Accepted for publication, July 23, 1935)

CORRECTIONS

In Vol 19, No 3, January 20, 1936 page 508, line 1, for and at time t , read "at time t "

On the same page, in the next to the last line "at fixed time t " should be followed by a comma

On page 518 line 3 the semicolon before 'The right hand graph' should be a period

On the same page, in the third line from the bottom of the page $\Delta I / \sigma_{\Delta I}$, should read $d\Delta I / d\sigma_{\Delta I}$

On page 536 in the heading of Table IV after 'with the standard deviations', insert (σ_1)

nitroprusside and ammonium hydroxide In crystalline egg albumin Arnold could detect SH groups only after denaturation Since egg albumin is a typical coagulable protein, and since none of the proteins of muscle have been isolated in a crystalline form, it was possible that the SH groups detectable in minced muscle in the isolated muscle proteins are due to the presence of denatured protein And since the proteins of muscle are supposed to be unusually unstable, presence

of denatured protein is perhaps to be expected and indeed, in the past, it has been believed that minced muscle contains some denatured protein (von Furth, 1919) In a previous paper it has been shown that in hemoglobin, a protein that is certainly in the native state, active SH groups are detectable There is accordingly also some reason to suppose that the proteins of muscle even in their native state may contain active SH groups

By studying the effect of hydrogen ion concentration on the number of active SH groups in the isolated muscle proteins and in the proteins as they occur in minced muscle, it is shown in this paper that most, and probably all, of the active groups detected are groups of proteins in the native state Investigation of the protein SH groups of muscle shows, then, that little, if any, denatured protein is present In the experiments described in this paper the numbers of active SH groups in all preparations of muscle proteins that have not been treated with agents known to denature other proteins are found to be far smaller than in the same preparations after the proteins have been treated with a denaturing agent This observation is not in agreement with the statements of Hopkins and Dixon (1922), Tunnichliffe (1925), and Hopkins (1925) that the number of protein SH groups in muscle preparations before and after they have been treated with boiling water is about the same, such differences as are observed being according to them attributable to the changed permeability of the muscle fibers

Although the SH groups of muscle proteins change considerably on denaturation, a study of the denatured muscle proteins may be of some significance, for it can be shown that in them the ratio of SH to S-S groups is much higher than that found in the mixed denatured proteins of other tissues, with a single exception—the crystalline lens of the eye

Comparison between the SH and S-S Groups of the Denatured Muscle Proteins and of Proteins of Other Tissues

Skeletal muscles of the halibut, frog, and rabbit were used In each case SH groups of the mixed proteins denatured by trichloroacetic acid were estimated A number of muscle protein fractions—the albumins (myogens) of halibut and rabbit and the myosins of frog and rabbit—

were isolated, denatured by trichloroacetic acid, and their SH groups estimated. The numbers of SH groups were, as in other denatured proteins, equivalent to the cysteine contents of the hydrolyzed proteins. The cystine contents, equivalent in denatured proteins to the numbers of S-S groups (Mirsky and Anson, 1934-35) of all preparations were estimated. In several cases the total number of S-S plus SH groups was estimated and found to be in fact equivalent to the cystine plus cysteine content. In each protein preparation the percentage of the total cysteine plus cystine content in the form of cysteine has been

TABLE I
Percentage of Total Cysteine Plus Cystine Found to Be Cysteine

Preparation	Cysteine content	Total cysteine plus cystine	Part of total present as cysteine	Cysteine content after reduction by thioglycolic acid
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Minced frog muscle	0.724	1.01	71	1.22
rabbit	0.79	0.97	82	
halibut	0.81	1.16	69	
Rabbit myosin	0.67	0.79	85	
Halibut myogen	0.95	1.18	80	
Salivary gland of ox	0.2	1.145	0.14	
Gray matter of brain of ox	0.2	1.12	0.18	
Crystalline lens	1.24	1.105	100	
Liver of dog	0.21	1.1	19	
Kidney	0.27	1.0	27	
Pancreas	0.14	1.7	8.1	16.9
Testicle	0.12	0.71	16.9	
Finger nails—man	0	10.3	0	

calculated and found to lie between 69 and 85 per cent. The amounts of cysteine and also of the total cysteine plus cystine have been estimated in the mixed proteins of a number of tissues and the percentages of the totals in the form of cysteine were found to be as follows: liver 19 per cent, kidney 27 per cent, pancreas 8.1 per cent, testicle 16.9 per cent, salivary gland 0.14 per cent, gray matter of the brain 0.18 per cent, finger nails 0 per cent, crystalline lens 100 per cent. With the exception of the crystalline lens, the percentages of cysteine were all distinctly lower than that found in skeletal muscle.

SH Groups of Muscle Proteins That Have Not Been Treated with Reagents Known to Denature Them

The method of estimation was to treat the protein with iodoacetate, which has been shown to react with active SH groups of proteins, estimate the cysteine content of the hydrolyzed protein, and compare it with the cysteine content of a sample of the protein that has not been treated with iodoacetate. The decrease in cysteine content of the protein treated with iodoacetate is a measure of the number of its active SH groups.

1 Mixed Proteins Present in Minced Muscle

It has been found that the SH groups of a protein can be activated by increasing the pH or at a constant pH by denaturing the protein. The protein SH groups of minced rabbit muscle can also be activated by raising the temperature (in a range below the coagulation temperature), and on cooling, activation is reversed. The SH groups of other proteins are probably affected by temperature in the same way.

The number of active protein SH groups in minced frog and rabbit muscle increases as the pH rises, as shown in Fig. 1. There is a certain similarity in the behavior of these groups and those of a well defined native protein, hemoglobin. As in the case of hemoglobin, the groups activated by a rise in pH become inactive again when the pH is restored to its original level.

There is also a difference in the behavior of the groups of hemoglobin and of the proteins in minced muscle. In hemoglobin, as the pH falls, a point is reached (pH 6.8) at which no groups are active in the native protein but at which all the groups of the denatured protein are active. In the proteins of minced muscle a plateau in the curve is reached as the pH falls. There is hardly any change in the number of active SH groups between pH 8.0 and 7.3. Below pH 7.3 not all of the groups even of the denatured protein react with iodoacetate. There are two possible explanations of this plateau: (1) that the SH groups active between pH 7.3 and 8.0 are those of denatured protein present in the minced muscle and that only those groups that become active when the pH rises above 8.0 are groups of native protein, (2) that the curve of the *native* proteins of muscle differs from that of hemoglobin.

A study of the SH groups of the isolated muscle protein fractions shows that the second explanation is more probable

2 *Myosin and Myogen*

In both of these fractions the behavior of SH groups is similar to that of minced muscle. In the case of myosin no decision can be made

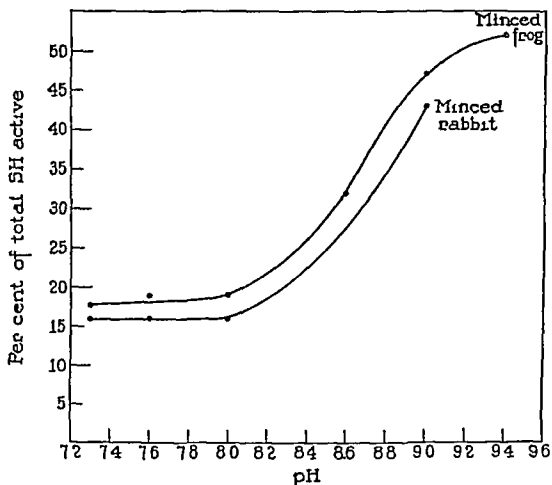


FIG 1 The relation between pH and the number of active SH groups in the proteins of muscle

between the two explanations either of which would explain the phenomenon of minced muscle for there is no evidence that myosin is a pure native protein free of a certain admixture of denatured protein. In the case of myogen, however, the method of preparation used makes it highly unlikely that any denatured protein is present. In the usual way of preparing myogen, by dialysis of the juice expressed from muscle, there is no reason to suppose that if denatured

protein were present in the original material, it would have been eliminated in the final product. The myogen used in the present investigation was prepared by extracting minced muscle with half-saturated ammonium sulfate and then precipitating the extracted protein by further addition of ammonium sulfate. Since no known denatured protein is soluble in half-saturated ammonium sulfate, even in the presence of native protein, it is unlikely that denatured protein is present in this preparation of myogen. Even after myogen has stood in the kind of solution used for detecting SH groups, no denatured protein can be found on half-saturating the solution with ammonium sulfate. The number of active SH groups in myogen varies with pH substantially in the same way as do the SH groups of myosin and of the proteins in minced muscle. Since there is no denatured protein in the preparation of myogen it is not necessary in explaining the effect of pH on SH groups to suppose there is any denatured protein in the preparations of myosin and minced muscle. The simplest explanation of the known facts is that there is little, if any, denatured protein in freshly minced muscle.

3 *The Experiments of Hopkins and Dixon, Tunncliffe, and Hopkins*

The muscle preparation used by these authors was one that after mincing was thoroughly extracted with water. Activity of protein SH groups was estimated (usually at pH 7.6) by adding glutathione and measuring the oxygen uptake of the system, or by adding oxidized glutathione and measuring the amount of reduced glutathione formed. They found that when muscle that had been simply washed with water was compared with muscle that was also treated with boiling water (the "thermostable preparation") or with alcohol that

"The reaction proceeds somewhat more smoothly in the case of the heated preparation, but within the limits of experimental error the total reduction is the same in both cases. The alteration in permeability of the preparation due to the heating would in all probability account for the greater ease of reduction in the case of thermostable preparations" (Tunncliffe). "A corresponding amount of the original washed muscle without any further treatment is itself capable of the same quantitative uptake (that is, as the thermostable residue), but to establish a sufficiently active system within the swollen and somewhat impermeable fibres of the preparation a relatively high concentration of glutathione in the external fluid is necessary" (Hopkins). "There seems to be little doubt that the effect of the preliminary treatment is on permeability. Heat and treatment with alcohol seem to influence this on similar lines" (Hopkins and Dixon).

TABLE II
SH Groups of Muscle Proteins before Treatment with Denaturing Reagents

Preparation	II										III				
	Cysteine in untreated protein		Cysteine content after reaction with iodoacetate at pH								Percentage of total SH active at various pHs, $\frac{I-II}{I}$				
	0.85	0.73	0.73	0.80	0.67	0.75	0.80	0.67	0.75	0.80	0.86	0.88	0.90	0.94	
Mixed frog muscle	0.85	0.70	0.69	0.69	0.59	0.45	0.37	0.26	0.17	0.19	0.32	0.47	0.52	0.65	
"	0.73	0.59	0.59	0.44	0.48	0.46			0.26	0.19	0.38	0.35			
"	0.73	0.54*	0.67	0.66	0.66	0.46			0.26	0.16	0.16	0.43			
"	0.80	0.67	0.66	0.66	0.66	0.46			0.26	0.16	0.16	0.43			
"	0.75	0.64†	0.67	0.66	0.66	0.46			0.26	0.16	0.16	0.43			
halibut	0.80	0.51	0.36	0.36	0.35	0.26	Very low		0.26	0.46	0.48	0.61	Very high		
Rabbit myosin	0.67	0.36	0.36	0.35	0.35	0.26	Very low		0.26	0.46	0.48	0.61	Very high		
Residue after ex rabbit myosin	0.73	0.51	0.51	0.51	0.51	0.68	0.67		0.68	0.30	0.30	0.19	0.20		
Halibut myogen	0.84	0.75	0.74	0.72	0.72	0.59	0.60		0.59	0.12	0.14	0.20	0.19		
"	0.74	0.62	0.60	0.61	0.61	0.59	0.60		0.59	0.16	0.18	0.20	0.19		

* Reaction with iodoacetate for 2 hrs 0.57
3 0.54
5 0.54
6 " 0.53

† Reaction at 20° 0.67
" " 36° 0.55

‡ Reaction at 20 0.64
" 36° 0.52

After treating all these preparations with CCl_3COOH and then allowing them to react with iodoacetate, no cysteine was found in any of the hydrolysates

Mixed Frog Muscle Washed with Distilled Water

Cysteine content of untreated preparation	Pe cent
" after reacting with K ferriocyanide at pH 7.6 for 1½ hours	0.65
" " " " " "	0.33
" " " " " "	0.31
" " " " " "	None

The experiments described in this paper show that treating muscle with a denaturing agent increases the number of active SH groups, and this effect of the denaturing agent is on the muscle proteins and not on the permeability of the muscle fiber. The evidence may be briefly summarized.

1 Iodoacetate reacts with more SH groups in minced muscle that has been treated with trichloroacetic acid than in untreated muscle, and the same difference is obtained whether the reaction with iodoacetate proceeds for 3 or for 6 hours, indicating that the reaction has gone to completion and that permeability is not a limiting factor.

2 Muscle extracted with water has lost none of its myosin. When myosin is extracted with a salt solution a similar difference between the number of its active SH groups before and after denaturation is observed as in the proteins of washed muscle. In this case there can be no question of permeability.

3 Due to the supposed absence of an effect of a denaturing agent on the SH groups of washed muscle Hopkins suggested that "the less soluble among tissue proteins share some special character with blood proteins rendered insoluble by denaturation." Even after all the myosin in minced muscle is extracted so that only the least soluble proteins of muscle remain, it is found that trichloroacetic acid has the same effect on the SH groups of these proteins that it has on all other muscle proteins, and indeed on all native proteins.

4 In the experiments of Tunncliffe and of Hopkins an oxidizing agent, oxidized glutathione, was used to react with sulfhydryl groups. If a strong oxidizing agent, potassium ferricyanide, is used in our experiments, the results obtained are the same as when iodoacetate is used. When untreated washed muscle and washed muscle that has been treated with trichloroacetic acid are mixed with ferricyanide, it is found that in the former far fewer SH groups are oxidized than in the latter, and the same result is obtained whether the reaction proceeds for $1\frac{1}{2}$ or for 4 hours.

EXPERIMENTAL

Protein Preparations

Skeletal muscle of the halibut, southern bull frog (*Rana catesbeiana*), and rabbit were used. The halibut muscle, obtained frozen from the Birdseye Company, was minced in a meat-grinder while frozen. The frogs were killed by being cut

in two by a single blow with a heavy cleaver across the spine, just below the heart. The rabbits were killed by injecting air in their veins. The hind legs of both animals were perfused with Ringer's solution to remove the blood. After dissecting the muscles they were passed three or four times through a meat grinder, containing a plate with fine holes.

Myogen was prepared from minced rabbit and halibut muscle. To the minced muscle was added four times its volume of half-saturated $(\text{NH}_4)_2\text{SO}_4$ and then its own volume of saturated $(\text{NH}_4)_2\text{SO}_4$. The mixture was gently stirred for 2½ hours and filtered. Enough solid $(\text{NH}_4)_2\text{SO}_4$ was dissolved in the clear filtrate to precipitate the protein completely (about 50 gm for 140 cc.) After standing for several hours the suspension was filtered and the next day the precipitated protein was scraped off the filter paper. All operations were carried out in a cold room at about 4°C.

Myosin was prepared from minced frog and rabbit muscle by Edsall's method (Edsall, 1930). After all the myosin had been extracted the residue was used in some experiments.

Minced muscle was washed with water in a somewhat different manner from that described by Hopkins. The procedure will be described in another paper.

For minced liver, kidney, pancreas, and testicle the tissues of a dog were used. The dog, after fasting for 48 hours, was perfused with Ringer's solution under local anesthesia (novocaine) until practically all blood was removed. Salivary glands, brains (gray matter) and crystalline lenses were obtained from steers at the slaughter house. Human finger nails were used. It was necessary to free the salivary gland of blood. This was done by washing the minced tissue with large amounts of cold distilled water, to which was added one tenth its volume of M/10 pH 5.0 acetate.

Cysteine and Cystine Contents of Mixed Tissue Proteins and of the Isolated Protein Fractions of Muscle

In the various tissues the mixed proteins were denatured by treating them with trichloroacetic acid. 5 gm (wet weight) of minced tissue were ground in a mortar with 50 cc of 5 per cent trichloroacetic acid. The mixture was diluted to 250 cc with 5 per cent trichloroacetic acid, stirred and then centrifuged. The supernatant fluid was discarded, and the precipitate washed four times with 250 cc of 5 per cent trichloroacetic acid. Myosin and myogen were denatured by adding to 225 cc of the protein solution, containing from 0.5 to 1.0 gm of protein, 15 cc of a concentrated trichloroacetic acid solution (the acid dissolved in an equal weight of water). One portion of each protein sample was dehydrated with acid acetone, dried, hydrolyzed, and the cysteine content of the hydrolysate estimated. Another portion was oxidized with hydrogen peroxide to convert any cysteine present into cystine. Estimation of the cystine content of the hydrolysate of the oxidized protein gave the total amount of cysteine plus cystine originally present in the protein. SH groups were estimated by the indirect method (Mirsky and

Anson, 1934-35) using iodoacetate to react with SH groups. In this method the difference in cysteine content of the protein treated with iodoacetate and that not treated indicates the number of SH groups that reacted with iodoacetate. The preparations of denatured muscle proteins after being treated with iodoacetate contained no cysteine, which showed that the numbers of SH groups in the denatured muscle proteins were equivalent to the cysteine contents of the hydrolysates of the untreated proteins.

Equivalence between Cystine Plus Cysteine Content and Number of S-S Plus SH Groups of Denatured Muscle Proteins

The total number of groups was estimated by reducing the denatured protein with thioglycolic acid, which converts any S-S groups present into SH groups. The number of SH groups is estimated by hydrolyzing the protein and measuring its cysteine content. This was found to be equal to the cystine content of oxidized protein.

Active SH Groups of the Mixed Proteins in Minced Muscle

1 *Reaction with Iodoacetate*—5 gm of freshly minced frog or rabbit muscle were ground in a mortar with 80 cc of an M/2 buffer and to the mixture were added 15 cc M/5 iodoacetate (iodoacetic acid neutralized to phenol red with sodium hydroxide). The mixture, transferred to a flask, stood at 20° with occasional agitation for 3 hours, when 100 cc of water and 20 cc of concentrated trichloroacetic acid were added. The mixed proteins were then washed with 5 per cent trichloroacetic acid to free them of iodoacetate and other salts. Under the conditions of the reaction—time, temperature, minimum pH, concentration of iodoacetate—all the SH groups of the denatured proteins in minced muscle react with iodoacetate.¹

The reactions with iodoacetate of myogen and myosin were carried out in the same manner. The precipitate of myogen in ammonium sulfate was dissolved by adding water. To 7 cc of the solution, containing about 0.5 gm protein, were added buffer and iodoacetate. In the case of myosin the bulky precipitate in ammonium sulfate was dissolved directly in the buffer, about 0.4 gm of protein being used.

The buffer solutions used were $K_2HPO_4 - KH_2PO_4$ of pH 7.3 to 8.0 and $NaOH - H_3BO_3$ of pH 8.0 to 9.6.

The time of the reaction was varied from 2 to 6 hours. After 3 hours no further change occurred.

¹ In one experiment iodoacetate of twice the concentration was used, and a slightly larger number of SH groups of native protein reacted with it than did with the iodoacetate which was sufficiently concentrated to react with all the SH groups of denatured protein.

2 *Estimation of the Groups Reacting with Iodoacetate*—The decrease in cysteine content of a protein treated with iodoacetate is a measure of the number of its SH groups that react with iodoacetate. After reacting with iodoacetate all proteins were washed, dehydrated with acid acetone, dried, hydrolyzed and the cysteine content of the hydrolysate estimated. The cysteine content of another sample of each protein preparation that had not been treated with iodoacetate was estimated. The difference in cysteine content between treated and untreated protein was the quantity of cysteine equivalent to the number of SH groups that reacted with iodoacetate.

3 *Reversibility of the pH Effect*—The effect of pH on the activity of SH groups was fully reversible. 8 gm. of minced frog muscle were placed in 40 cc. of a pH 9.4 buffer. The flask was filled with nitrogen and stood at 20° for 2 hours. The mixture was then divided into two parts. In one part the cysteine content was estimated and found to be unaltered. To the other part were added 50 cc. of a $\times 0.1$ pH 7.3 buffer and 15 cc. of iodoacetate. The number of active SH groups was that usually found at pH 7.3.

4 *Effect of Temperature*—Rabbit muscle at pH 7.3 was treated with iodoacetate at both 20° and 36°. More SH groups reacted at 36°. The effect of temperature is reversible. Minced muscle was mixed with 85 cc. of buffer solution to which a trace of iodoacetate (0.5 cc. of a $\times 10$ solution) was added because it has been stated that small amounts of iodoacetate inhibit intracellular proteolytic enzymes² (Maschmann 1933). The flask, filled with nitrogen, remained at 36° for 2 hours. The mixture was cooled to 20° and to it were added 15 cc. of $\times 5$ iodoacetate. The same number of groups reacted with iodoacetate as did in muscle that had not been warmed to 36°.

5 *Reaction of SH Groups with Ferricyanide*—Minced frog muscle that had been washed with water was used about 0.7 gm. (dry weight) for each experiment. To denature the material it was suspended in 225 cc. of water and 20 cc. of concentrated trichloroacetic acid were added. After centrifuging the precipitate was washed with 250 cc. of water. It was then stirred in 225 cc. of water to which 25 cc. of a pH 7.6 $\times 1$ KH_2PO_4 - K HPO_4 buffer were added and centrifuged once more. The precipitates of native and denatured material were suspended in 100 cc. of water to which 5 cc. of the pH 7.6 buffer and 10 cc. $\times 2$ potassium ferricyanide were added. The mixtures were kept at 20° with occasional agitation for 1½ hours. To each flask were added 100 cc. of $\times 20$ KH_2PO_4 and the suspensions were centrifuged. The precipitates were then washed with $\times 100$ pH 6.8 phosphate buffer by repeated stirring and centrifuging until the yellow color of ferricyanide disappeared. They were freed of salt by washing with trichloroacetic acid and then they were dehydrated, dried, hydrolyzed and the cysteine contents of the hydrolysates were estimated.

² The quantity added was not enough to react with a detectable number of protein SH groups.

SUMMARY

1 In the denatured proteins of skeletal muscle, the ratio of SH to S-S groups is higher than in the mixed denatured proteins of other tissues, with a single exception—the proteins of the crystalline lens

2 The number of active SH groups in the proteins of minced muscle or in any of the protein fractions of muscle is only a fraction of the number found after the proteins have been treated with a denaturing agent

3 The SH groups of the native proteins of muscle are activated by a rise in pH

4 The relation between pH and number of active SH groups in the proteins of minced muscle and in the various protein fractions of muscle shows that little, if any, denatured protein is present in minced muscle

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THE CHANGE IN STATE OF THE PROTEINS OF MUSCLE IN RIGOR

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(Accepted for publication, July 23, 1935)

When a muscle contracts or goes into rigor, the myosin within it becomes changed, the change being detectable as a loss in solubility. The difference between the state of myosin in rigor and the state of myosin which has been rendered insoluble by a typical denaturing agent, such as heat or acid, is described in this paper.

Although the altered solubility of muscle proteins in rigor was observed 27 years ago by Saxl, it has been the subject of very few investigations. Saxl thought that the change in solubility was in the myogen (albumin fraction) of muscle, and it was not until 1924 (Howe), or more decisively until 1933 (Weber and Meyer) that this mistake was corrected and it was shown that the change was in myosin. In 1930 Deuticke observed that a similar, or perhaps identical, change occurs in myosin during contraction, and that on recovery the change is reversed. By loss of solubility, it is not meant that myosin precipitates within the muscle. There is indeed evidence that even in resting muscle myosin is not in solution (Smith, 1934). The myosin of active muscle or of muscle in rigor is modified so that when the structure of the tissue is destroyed this protein does not dissolve in a salt solution in which the myosin of resting muscle does dissolve. The scale of the phenomenon is striking, in rigor 30 per cent of the total protein in muscle becomes changed.

Many proteins are modified when exposed to heat, acid, alkali, alcohol, urea, salicylate, ultraviolet light, surface forces, or other agents so that they are no longer soluble under conditions under which the unchanged proteins are soluble. The change is known as denaturation. Because of the superficial resemblance, the change in solubility that occurs in myosin has also been referred to as "denatura

tion" (Weber and Meyer, von Baeyer and von Muralt) It is now possible to define denaturation more precisely than could be done formerly (Mirsky and Anson, 1935-36) When a protein is acted upon by any one of the agents just mentioned, there occurs both a loss of solubility and an activation of all its S-S and SH groups The two changes are so closely linked that they must be considered to be integral parts of the same process Denaturation has hitherto been defined by the change in solubility alone, but since all known denaturing agents activate S-S and SH groups at the same time that they render the protein insoluble, denaturation is more completely and precisely defined in terms of both of these changes

When myosin is treated with a reagent that denatures other proteins the changes characteristic of denaturation are observed But when in rigor myosin loses its solubility it is shown by the experiments described in this paper that no activation of SH groups occurs This change in myosin is, therefore, distinctly different from the change in it caused by a typical denaturing agent The difference is clearly emphasized when myosin rendered insoluble in rigor is treated with a denaturing agent, acid No change in solubility is now observed, but the SH groups of myosin are activated just as they are when native soluble myosin or any other native protein is treated with an excess of acid Protein coagulation, loss of solubility, can therefore occur in two different ways

Coagulation of protein in which loss of solubility occurs without the activation of SH and S-S groups characteristic of denaturation is not limited to muscle This change can be observed in the proteins of other tissues under certain conditions to be described in another paper

Observations of the SH groups of myosin are useful in interpreting some recent experiments of Astbury and Dickinson on the crystallographic properties of myosin In his studies of the crystal pattern of wool, as revealed by the x-ray diffraction method, Astbury finds that certain changes occur when wool is treated with steam When myosin is exposed to steam similar changes are detected Astbury and Dickinson suggest that the myosin in muscle undergoes this change in the course of muscular activity When a fiber of myosin, such as was used by Astbury and Dickinson is exposed to steam for only 2 seconds, I find that all its SH groups have been activated, that the

myosin has been denatured. The change in myosin known to occur when muscle becomes active is therefore distinctly different from the change that Astbury and Dickinson suppose takes place. If a change in myosin like that due to steam occurs at all in muscle, it occurs in such a minute quantity of myosin that it is not detectable by the present methods for estimating protein SH groups.

EXPERIMENTAL

The experiments were on the skeletal muscle of the southern bull frog (*Rana catesbeiana*). Rigor was produced, as described by Lundsgaard, by the injection of iodoacetate in a dorsal lymph sac. 10 cc. of M/10 iodoacetate (iodoacetic acid neutralized with sodium hydroxide) were injected. The onset of rigor became apparent in 2 or 3 hours, and the frog was then left in a cold room at 8° overnight. Next morning its limbs were stiff.

The muscles of the hind leg were minced, and the number of active SH groups was estimated by the reaction with iodoacetate in precisely the same manner previously described for minced muscle prepared from resting muscle. The procedure for denaturing the proteins in minced rigor muscle is the same as that previously described for resting muscle.

The quantity of protein becoming insoluble in rigor was measured. Muscles at rest and in rigor were minced, and 15 gm. of each were transferred to 250 cc. centrifuge flasks. To each flask were added 200 cc. of cold 1.2 M KCl and 4 cc. M/1 K_2HPO_4 . The flasks were placed in ice mixtures and their contents stirred for 2 hours. After centrifuging the supernatant fluid of each flask was poured into a 500 cc. flask, and to the residue in each centrifuge flask were added 225 cc. 1.2 M KCl in which the minced muscle was re-extracted for 2 hours. To the total supernatant fluid of each extraction were added 30 cc. concentrated trichloroacetic acid (the acid dissolved in an equal weight of water) to precipitate the protein. This precipitate and the residue of extracted tissue, both in 250 cc. centrifuge flasks, were freed of salt by repeated washing with 5 per cent trichloroacetic acid, the washing fluid being removed by centrifuging. Each precipitate, in a 250 cc. centrifuge flask, was dehydrated by washing twice with acid acetone (1 cc. of concentrated HCl in 200 cc. of acetone), and the lipoids which remained were removed by washing twice with a mixture containing three parts of alcohol and one part of ether. The dry weight of the substance in each flask, which now consisted practically entirely of protein, was determined after drying to constant weight at 110°.

Fibers of frog myosin spread out in a thin sheet were exposed to steam for 2 seconds. On testing with nitroprusside and ammonium hydroxide an intense reaction was obtained indicating the presence of SH groups. The steamed fibers were treated with iodoacetate at pH 7.3 for 3 hours and were then found to give no test for SH groups. The preparation was completely denatured, for even after subsequent treatment with trichloroacetic acid it failed to give a test for

SH groups When a myosin fiber, which has not been exposed to steam is treated with iodoacetate and then with trichloroacetic acid an intense test for SH groups is obtained After treating with trichloroacetic acid it is important to wash the protein with $M/2$ pH 7.3 phosphate buffer before testing with nitroprusside

RESULTS

Extraction of Protein from 15 Gm. of Minced Muscle

	<i>Resting muscle</i>	<i>Muscle in rigor</i>
Weight of protein extracted	1.94 gm	1.0 gm
Weight of residual protein	0.93	1.61
	<hr/>	<hr/>
Total protein	2.87	2.61
Percentage of protein extracted	67.6	38.3

SH Groups of the Proteins in Minced Muscle

Muscle in rigor—I

- 1 Cysteine content of untreated muscle proteins 0.74 per cent
- 2 Cysteine content after treating with iodoacetate at pH 7.3 0.62 per cent
- 3 Percentage of total number of SH groups that react with iodoacetate, that is $\frac{(1)-(2)}{(1)}$ 15.8 per cent

Muscle in rigor—II

- 1 Cysteine content of untreated muscle proteins 0.75 per cent
 - 2 Cysteine content after treating with iodoacetate at pH 7.6 0.62 per cent
 - 3 Percentage of total number of SH groups that react with iodoacetate 17.6 per cent
- Average of the two experiments 17 per cent

Resting muscle

- Percentage of total number of SH groups reacting with iodoacetate at pH 7.3 and 7.6
- Average of several experiments taken from a previous paper 19 per cent

Rigor muscle denatured by trichloroacetic acid

- Cysteine content of mixed proteins 0.74 per cent

Cysteine content after treating with iodoacetate at pH 7.3	too low to estimate accurately
Percentage of total number of SH groups that react with iodoacetate	nearly 100 per cent

CONCLUSIONS

1 When myosin is exposed to a typical denaturing agent (acid) it becomes insoluble and its SH groups are activated

2 The same number of active SH groups is found in the soluble myosin of resting muscle as in the insoluble myosin of muscle in rigor. No activation of SH groups accompanies the formation of insoluble protein in rigor

3 When the insoluble myosin of muscle in rigor is treated with a denaturing agent its SH groups are activated

4 Protein coagulation as brought about by denaturing agents (heat, acid, alkali, alcohol, urea, salicylate, surface forces, ultraviolet light) is a distinctly different change from the coagulation of myosin brought about by the unknown agent in muscle

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ANOMALIES IN THE ABSORPTION SPECTRUM AND BLEACHING KINETICS OF VISUAL PURPLE

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I

Problem

In the bleaching of visual purple by light, Kühne (1879) had found that a yellow color appeared which was not a mere dilution of the reddish color of the original visual purple. This was confirmed by Garten (1907) whose spectrophotometric measurements showed an increase in absorption in the blue and violet at intermediate stages of bleaching, instead of the uniform decrease throughout the spectrum which should result if visual purple changed directly to a transparent, colorless condition. On continued illumination the blue absorbing substance finally disappeared, leaving the solution colorless.

Garten's work was directly opposed to the previous measurements of Kottgen and Abelsdorf (1896), who had found that visual purple solutions were bleached by light without giving rise to an intermediate yellow color and that, measured spectrophotometrically, the absorption decreased uniformly throughout the spectrum.

There is no reason to believe that the findings of any of these investigators are unreliable. Instead it is probable that some difference in material or method is responsible for the contradiction. If this is so, then it should be possible to duplicate both results under proper conditions. The experiments to be reported do precisely this, and offer an explanation of the discrepancies.

II

Methods

Visual purple extractions using Kühne's procedure as modified by Hecht (1921), were made from the retinas of frogs (*R. pipiens*) obtained from Alburgh

Vermont Ordinarily 20 or 30 frogs were dark-adapted at room temperature (16° to 24°C) for at least 8 hours and then, by the light of a 10 watt ruby lamp, their heads were cut off and dropped into 500 cc. of distilled water. The eyes, removed, were cut through with a razor blade just back of the lens. The retinas were lifted out as free as possible of the pigment layer by means of fine, curved forceps and placed in 8 to 10 cc. of distilled water. This retinal suspension was centrifuged at a moderate speed for 30 minutes and the supernatant liquid discarded. From 5 to 8 cc. of a 4 per cent purified bile salts solution were then added, and the packed retinas thoroughly stirred into suspension, and left at room temperature for 45 to 90 minutes. The suspension was then centrifuged at a high speed for 45 to 120 minutes, and the supernatant clear liquid was finally pipetted off from the packed debris and stored in a light-tight container at 0°C . The prolonged centrifuging at this point gave solutions which remained clear and stable longer than those centrifuged for shorter times.

The measurements were made with a Koenig-Martens spectrophotometer using two procedures. In one the absorption cell containing the solution was placed in one beam of the instrument, the other beam being free. In the other (used by Kottgen and Abelsdorf) the solution occupied one beam while an identical sample, fully bleached, was in the other. The readings obtained by the first method represent the sum of the absorptions of all the materials extracted from the retinas, plus the bile salts solution. The absorption spectrum of visual purple is then secured by subtracting the density of the fully bleached from that of the unbleached solution. The second method carries this subtraction out automatically. However, it is less reliable during partial bleaching since the supposedly fully bleached sample may undergo further change during the time that the experimental sample is being illuminated. The bulk of the data was therefore secured by the first method.

All the measurements are given in terms of density, which is defined as $\log I_0/I_t$, where I_0 is the incident light and I_t the transmitted light. The relation between the light incident on an absorbing solution and that which is transmitted is $I_t = I_0 e^{-\epsilon cd}$, where c is the concentration of the solution, d the depth, and ϵ is the extinction coefficient of the solute. In the form, $\epsilon cd = \log I_0/I_t$, the product ϵcd equals the density. If the absorption spectra are measured at different stages during bleaching, a comparison of the curves obtained will show whether c alone is changing or whether ϵ is changing also. In the latter case the appearance of some new substance is indicated during bleaching.

Since most of the measurements here reported were in the blue end of the spectrum, stray light was largely eliminated by interposing in both beams Wratten filter 47, which transmits only violet, blue, and a small fraction of green light. This filter also served the purpose of shielding the solution from unnecessary bleaching by the measuring light.¹

¹ An additional error, always present in measurements of light transmission of photosensitive substances, arises from the fact that the substance under investiga-

To increase the normally poor visibility at short wave lengths, use was made of a photoflood lamp to produce an intense light with a relatively high energy in the blue and violet. Later this was replaced by a specially focussed beam from a 100 watt concentrated filament lamp, which gave an equally high energy in the blue and lasted longer.

Density changes not due to illumination occur in solutions of visual purple. They are probably due to the growth of bacteria, making it difficult to prolong an experiment beyond a certain time and often affecting results even in a very short interval. The usual means of sterilization are not applicable to visual purple because of its instability, but it was found that a small amount of hexylresorcinol greatly delayed the density changes attributable to bacteria without perceptible effect on the visual purple itself. However, in none of the experiments reported here was this method used, since the prolonged centrifuging was adequate. Kühne mentions the use of a concentrated NaCl solution in this connection.

III

Seasonal Differences

If the complexities in the bleaching of visual purple are due to the formation of an intermediate substance, absorption spectra of mixtures of completely bleached and unbleached visual purple solutions should not show these complexities. The data² in Fig 1 record such measurements, and confirm this expectation. Absorption spectra of an identical sample were measured during illumination and in this case complexities in the blue and violet occurred as also shown in

tion may be affected by the light used for making a single measurement. Preliminary tests showed that a slight amount of bleaching of the solutions occurred through this cause. To reduce this small error to a minimum the experimental solution was stirred by periodical shaking and the number of measurements was reduced to the smallest required to give a dependable average value. Most of the experimental points represent an average of only three settings of the nicols and the data show this number to be adequate.

² In order to make the different figures more easily comparable the data plotted are the values for the density of the visual purple and other labile components of the retinal extract unless otherwise stated in the legend. They were obtained by subtracting the density of the fully bleached solution from the densities of the unbleached and partially bleached.

The values given in the Tables however, are the data as actually measured. Since the density of the fully bleached total solution is also given, the data as plotted in the Figures can be obtained by subtracting this fully bleached value from the other values in the Tables.

Fig 1 These data are representative of many,—all showing the same thing, it is significant that they were all made in the winter

During July, 1932, a visual purple extraction was made from frogs that had not been stored Absorption spectra of two samples of this extraction were measured during illumination, using the two-cell method of Kottgen and Abelsdorf Table I gives the original data, and Fig 2 (right side) the plotted curves, for one of these samples In

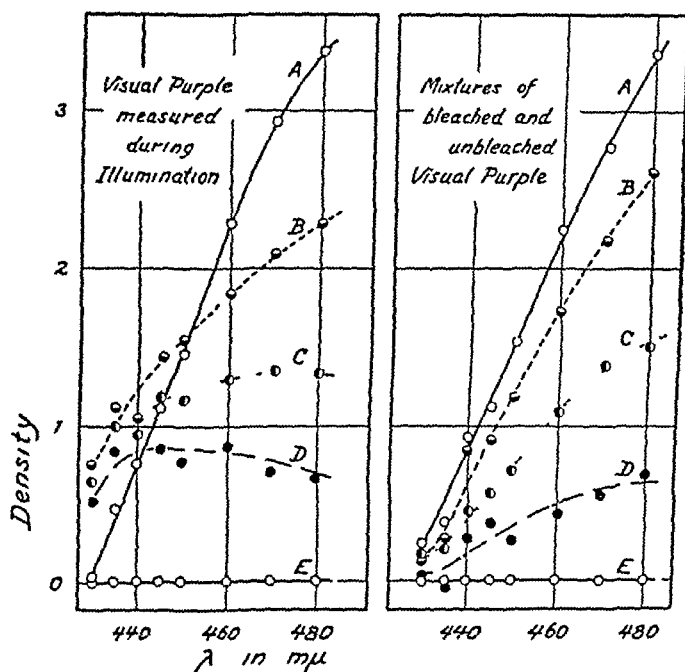


FIG 1 Comparison of absorption spectra of mixtures of fully bleached and unbleached visual purple solutions with absorption spectra measured during illumination The points plotted represent density of the labile components of the solution only

contrast to the curves of Fig 1, complexities in the blue and violet are almost lacking

The following winter it proved impossible, using hibernating frogs, to repeat the results just described, but in the spring and summer of 1933, with freshly caught frogs, absorption spectra measured at different stages of bleaching resembled those of the preceding summer (See Table I and Fig 2, left side)

The results of typical experiments during the winter of 1932-33 are

TABLE I
Visual Purple Extractions Showing the Seasonal Effect

λ in μ	Summer of 1932					
	Densities after illumination for various times in seconds (double cell method)					
	0 0	7 0	15 0	35 0	∞	
430	0 043	0 049	0 058	0 082	—	
440	0 122	0 109	0 094	0 070	0 030	
450	0 268	0 218	0 143	0 097	0 030	
460	0 378	0 281	0 174	0 070	0 030	
470	0 513	0 362	0 227	0 088	0 030	
485	0 586	0 425	0 236	0 073	0 024	
500	0 671	0 484	0 271	0 073	0 015	
515	0 647	0 456	0 246	0 055	0 021	
530	0 541	0 372	0 199	0 039	0 012	
545	0 368	0 249	0 137	0 027	0 003	
560	0 214	0 146	0 079	0 018	0 015	
580	0 079	0 061	0 030	0 003	0 000	
600	0 030	0 009	0 003	0 000	—	

λ in μ	Summer of 1933					
	Densities after illumination for various times in minutes					
	0 0	0 5	1 5	4 0	25 0	∞
430	1 003	1 024	1 046	1 085	1 068	0 997
440	1 035	1 003	0 987	0 997	0 966	0 907
450	1 074	0 987	0 946	0 902	0 892	0 827
470	1 194	0 987	0 864	0 787	0 744	0 699
500	1 246	0 936	0 736	0 616	0 601	0 571

λ in μ	Winter of 1932-33					
	Densities after illumination for various times in minutes					
	0 0	1 0	3 0	10 0	20 0	∞
435	0 936	1 056	1 172	1 254	1 200	0 794
445	0 986	1 042	1 112	1 170	1 086	0 670
455	1 076	1 080	1 090	1 078	0 986	0 573
470	1 185	1 080	1 000	0 903	0 818	0 451

λ in μ	Winter of 1932-33									
	Densities after illumination for various times in minutes									
	0 0	0 5	1 0	2 0	4 0	8 0	15 0	30 0	60 0	∞
430	1 672	1 809	1 847	1 900	1 942	1 900	1 822	1 797	1 716	1 553
440	1 601	1 704	1 750	1 761	1 738	1 700	1 631	1 572	1 525	1 368
450	1 591	1 631	1 631	1 661	1 610	1 544	1 456	1 431	1 368	1 266
465	1 601	1 581	1 525	1 507	1 415	1 338	1 273	1 226	1 175	1 102
480	1 581	1 499	1 423	1 353	1 220	1 168	1 097	1 085	1 029	0 982
500	1 499	1 360	1 266	1 150	1 019	0 966	0 921	0 902	0 864	0 855

shown in Table I and plotted in Fig 3 for comparison with those of the summer seasons. Whereas in the latter the density at $440\text{ m}\mu$ continually decreases, in the winter extractions there is at first an increase in density, even at $455\text{ m}\mu$. This increase in the blue-violet finally ceases, and with sufficient illumination a decrease occurs to a value equal to or less than the original density (*cf* Hecht and Chase, 1934)

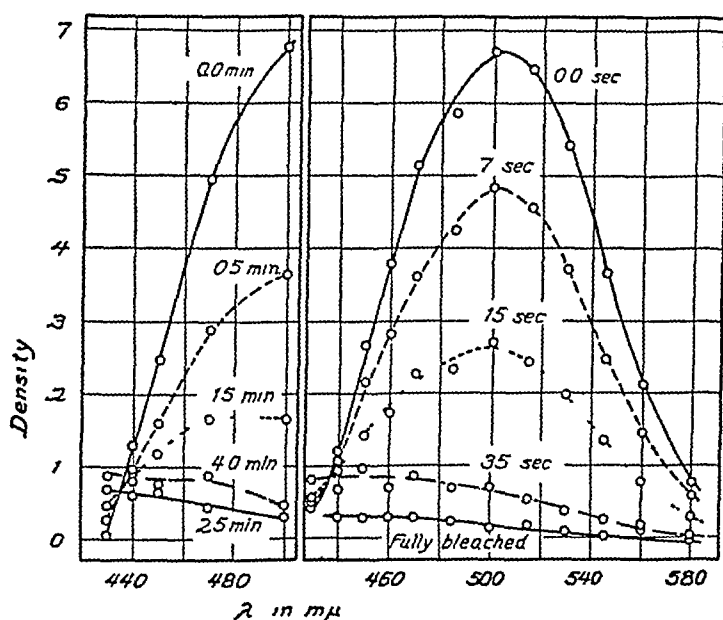


FIG 2 Absorption spectra measured during illumination of summer visual purple extractions. The data on the right were secured in the summer of 1932 while those on the left are of the summer of 1933. There is little evidence of complexities in the blue and violet. See Table I.

IV

Hydrogen Ion Concentration

The differences in behavior of summer and winter visual purple extractions can be imitated by varying the hydrogen ion concentration of solutions made from summer frogs. One experiment, selected from many similar ones, will show the typical results. To each of 3 equal volumes of visual purple there was added one-half its volume of $M/5$ buffer so as to yield a pH of 5.8, 7.0, and 9.3. The absorption spec-

trum was then measured at various stages in bleaching. The results are given in Table II and Fig. 4. At pH 5.8 illumination is accompanied by a greatly increased absorption in the blue and violet, which disappears upon prolonged exposure to the photo-flood lamp. At pH 9.3, on the other hand, there is no trace of new color during illumination. The sample buffered at pH 7.0 is intermediate. Measurements of an unbuffered winter extraction with natural pH of 6.1 are also

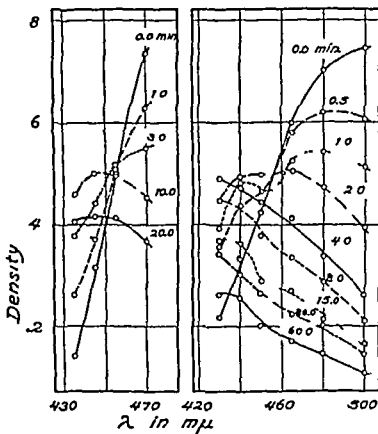


FIG. 3 Absorption spectra measured during illumination of typical winter visual purple extractions. Complexities in the blue and violet are very marked. See Table I.

shown in Fig. 4, they resemble those of the summer sample buffered at pH 5.8.

Upon illumination of visual purple solutions buffered at pH 5.3 the absorption increased greatly in the blue and violet and to some extent in the red, but this was largely due to the precipitation as suspension of some component of the solution, independent of light. Rendering the solution alkaline after illumination caused the disappearance of this suspension.

TABLE II

Absorption Spectra of Visual Purple Extractions Measured during Illumination at Various Hydrogen Ion Concentrations

Samples of a summer extraction were adjusted to pH 5.8, 7.0, and 9.3 by means of buffer solutions. The sample with pH of 6.1 was from an unbuffered winter extraction.

pH	λ in $m\mu$	Densities measured after illumination for various times in seconds				
		0 0	7 0	20 0	60 0	∞
9.3	430	0.635	0.601	0.545	0.495	0.449
	440	0.626	0.567	0.495	0.405	0.375
	450	0.646	0.566	0.459	0.361	0.315
	470	0.744	0.612	0.456	0.310	0.255
	500	0.770	0.605	0.436	0.255	0.208
	530	0.616	0.498	0.358	0.221	0.190
	550	0.419	0.345	0.271	0.205	0.177
	600	0.171	0.165	0.159	—	0.149
5.8	430	0.792	0.864	0.921	0.966	0.783
	440	0.792	0.841	0.869	0.878	0.679
	450	0.818	0.836	0.832	0.783	0.593
	470	0.897	0.836	0.744	0.624	0.460
	500	0.921	0.779	0.616	0.436	0.339
	530	0.715	0.589	0.449	0.316	0.268
	550	0.477	0.419	0.342	0.262	0.240
	600	0.108	0.205	0.205	—	0.193
7.0	430	0.757	0.779	0.787	0.824	0.707
	440	0.766	0.744	0.740	0.711	0.631
	450	0.792	0.749	0.707	0.635	0.549
	480	0.926	0.809	0.659	0.463	0.415
	500	0.961	0.783	0.608	0.382	0.352
	520	0.859	0.667	0.516	0.300	0.274
	550	0.509	0.415	0.332	0.218	0.218
	600	0.199	0.183	0.174	0.168	0.168
6.1	430	0.921	0.966	1.045	1.068	0.873
	440	0.901	0.916	0.931	0.936	0.756
	450	0.921	0.892	0.873	0.836	0.655
	470	0.971	0.897	0.787	0.675	0.505
	500	1.008	0.850	0.691	0.506	0.372
	530	0.753	0.659	0.488	0.355	0.281
	550	0.491	0.439	0.368	0.284	0.243
	600	0.230	0.227	0.218	0.208	0.199

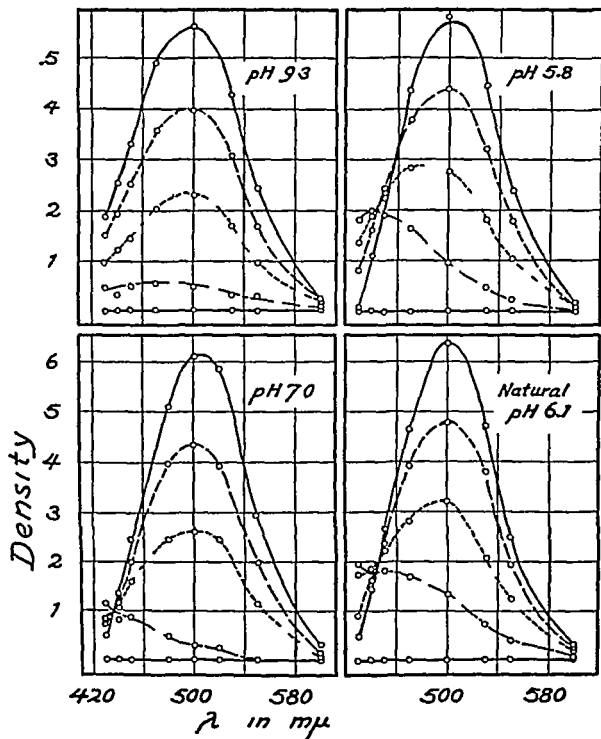


FIG 4 Absorption spectra during illumination of visual purple solutions of various hydrogen ion concentrations. The solution of pH 6.1 was an unbuffered winter extraction. The other pH values were produced by buffering three samples of a summer visual purple extraction. See Table II.

Since three buffer systems, phthalate, phosphate and borate KCl, were required to cover the range of hydrogen ion concentrations between pH 5.3 and 10.0, measurements were made of two samples of visual purple at pH 5.8, using phos

phate buffer in one and phthalate in the other. The identical results obtained showed that the observed effects were due to hydrogen ion concentration rather than composition of the buffers. The phosphate and borate-KCl buffers were similarly compared at pH 8.0.

Nakashima (1929) in a study of the effects of a number of substances upon unbleached visual purple in solution reported that acids and alkalis both caused the reddish color to disappear. In the case of the acid a distinct yellow was produced. He concluded that the conflicting results in the literature can be explained on the basis of differences in hydrogen ion concentration. Our data resulting from the use of buffers between pH 5.8 and 10.0 are essentially different from those of Nakashima in that decomposition of the visual purple in acid or alkaline buffer never occurred in the dark, illumination being always necessary. This would indicate that the hydrogen ion concentrations used by Nakashima may have been outside of the physiological range so that use of his results to explain the discrepancies in the literature seems unwarranted. His conclusions, however, are supported by the data presented here.

The yellow color in winter visual purple solutions during illumination might be due to increase in hydrogen ion concentration of the solution. The experiments with buffered visual purple extractions indicate that if this is the case a decrease in pH of about 1.2 units should accompany the bleaching of unbuffered winter extractions. Change of pH of such magnitude should be easily measurable.

An increase of acidity of the dark-adapted retina upon illumination has been found by many investigators, using indicator and titration methods (Kühne, 1879, Lodato, 1891, Rochat, 1904, Angelucci, 1905, Dittler, 1907). Lange and Simon (1922) found that the phosphoric acid content of the retina increased upon illumination. However, no such change has been demonstrated in the case of visual purple solutions.

The pH of winter visual purple extractions was measured during illumination, using both the hydrogen and quinhydrone electrode. Although a slight potential drift occurred throughout the measurements, there was no change coinciding with the short period of strong illumination. Measured values of pH lay between 5.9 and 6.2 for four samples of solution.

Since the pH of the winter extraction apparently remains constant during illumination, it is necessary, if pH be considered responsible for the seasonal variation, for a summer extraction to have a value about 1.2 units higher in order that yellow color shall be slight during bleach-

ing However, the pH of a visual purple extraction made from summer frogs in July, 1935, was found to be the same as that of the winter extractions previously measured, so that changes in hydrogen ion concentration cannot be considered responsible for the seasonal variation, although such changes, artificially produced, have similar effects and have probably contributed to the contradictory results reported in the past

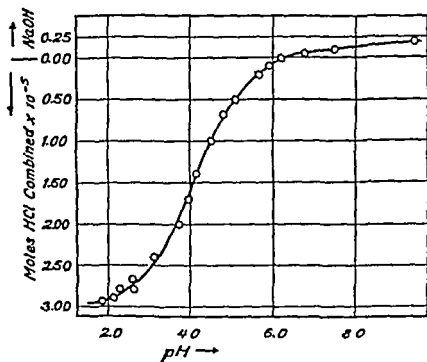


FIG 5 Titration curve of the purified 4 per cent bile salts solution as used in making visual purple extractions. The pH measurements were made with a Clark micro hydrogen electrode vessel the values of 1.0 cc. samples being determined after addition of varying quantities of HCl and NaOH. The curve shows no buffering capacity above pH 6.0 and a natural pH, for the aqueous solution, of about 6.2.

A measurement of the titration curve of a 4 per cent purified bile salts solution showed that its natural pH is 6.1 and that practically no buffering capacity exists at this pH and above (see Fig 5). Consequently, traces of base present could exert an effect sufficient to prevent the appearance of yellow color during bleaching. The results of Kottgen and Abelsdorf might therefore be explainable in this way, since bile salts were used in their procedure.

V

Visual Yellow

Visual yellow has assumed importance in the literature as a photic decomposition product of visual purple, essential to the process of vision (Garten, 1906). The experiments of the last section show that the yellow color occurs during illumination in acid but not in alkaline buffer. If the production of this yellow color during illumination in acid buffer is really dependent upon light, it should be impossible to cause its appearance in partially bleached solutions in the dark. The following experiment was designed to test this point.

To 7.0 cc of winter visual purple extraction was added 3.5 cc of $M/15$ pH 9.3 borate-KCl buffer. Before illumination two 1.0 cc samples were removed. To one was added 0.4 cc of $M/5$ pH 9.3 borate-KCl buffer and to the other the same amount of $M/5$ phosphate buffer pH 6.2, so that the resulting pH of the mixture was 6.6. These two samples were then stored in the dark at room temperature for 2 hours to allow time for any change to occur. The original mixture was exposed to an intensity of 15 foot candles for 20 minutes and two more 1.0 cc samples were withdrawn, treated, and stored in the same way. This procedure was repeated at 6.5, 11.5, and 18.0 minutes of bleaching.

Table III and Fig. 6 contain measurements of the absorption spectra of these ten samples. There is only a slight difference in density between the unilluminated samples buffered at the two pH values. After illumination, however, a pronounced difference appears in the dark. Since the yellow color does not necessarily accompany illumination but can be developed in the dark by increasing the hydrogen ion concentration of partially bleached solutions, visual yellow, so called, should not be regarded as essential to vision.

Since a yellow color develops in acid-buffered visual purple solutions as bleaching occurs, while no yellow color appears in alkaline-buffered solutions, the two apparently different types of visual purple bleaching in acid and basic buffers are merely due to the method of measurement which utilizes light transmission by the solution and consequently fails to differentiate between density differences due to visual purple concentration and density differences caused by changes in other colored components of the solution. Direct chemical estimation of visual purple concentration during illumination, if it were possible, should be free from such influences.

TABLE III

Visual Purple Solution Buffered at pH 9.3 and Illuminated

Samples removed at intervals during illumination and adjusted to pH 6.6 and 9.3 in the dark. Absorption spectra measured after remaining in the dark for 2 hours

λ in $m\mu$	Densities measured after illumination for various times in minutes									
	0 0		2 0		6 5		11 5		18 0	
	pH 6.6	pH 9.3	pH 6.6	pH 9.3	pH 6.6	pH 9.3	pH 6.6	pH 9.3	pH 6.6	pH 9.3
430	0.749	0.719	0.766	0.683	0.792	0.659	0.783	0.643	0.805	0.631
440	0.689	0.651	0.667	0.593	0.671	0.556	0.655	0.527	0.667	0.513
450	0.663	0.620	0.631	0.552	0.601	0.488	0.563	0.453	0.560	0.432
460	0.655	0.608	0.608	0.541	0.541	0.449	0.495	0.398	0.491	0.375
480	0.663	0.631	0.589	0.545	0.481	0.412	0.425	0.345	0.402	0.323
500	0.639	0.620	0.556	0.513	0.425	0.378	0.365	0.306	0.342	0.281

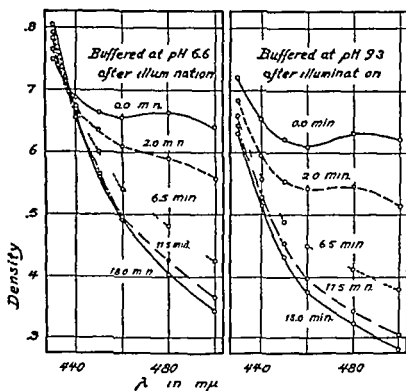


FIG. 6 Absorption spectra of unbleached and partially bleached samples of an alkaline buffered visual purple solution adjusted to pH 6.6 and 9.3 after illumination. A decomposition product is demonstrated whose absorption in the blue and violet is greater at low than at high pH. The color of this decomposition product depends upon the hydrogen ion concentration and not upon illumination. The density values plotted are those of the whole solution. See Table III.

VI

Temperature

15 minutes' exposure to a photoflood lamp 6 inches above the solution cooled by ice water, causes the disappearance of most of the yellow color which occurs when winter visual purple extractions (unbuffered or buffered at pH 6.2) are partially bleached. If the disappearance of yellow color represents a chemical rather than pho-

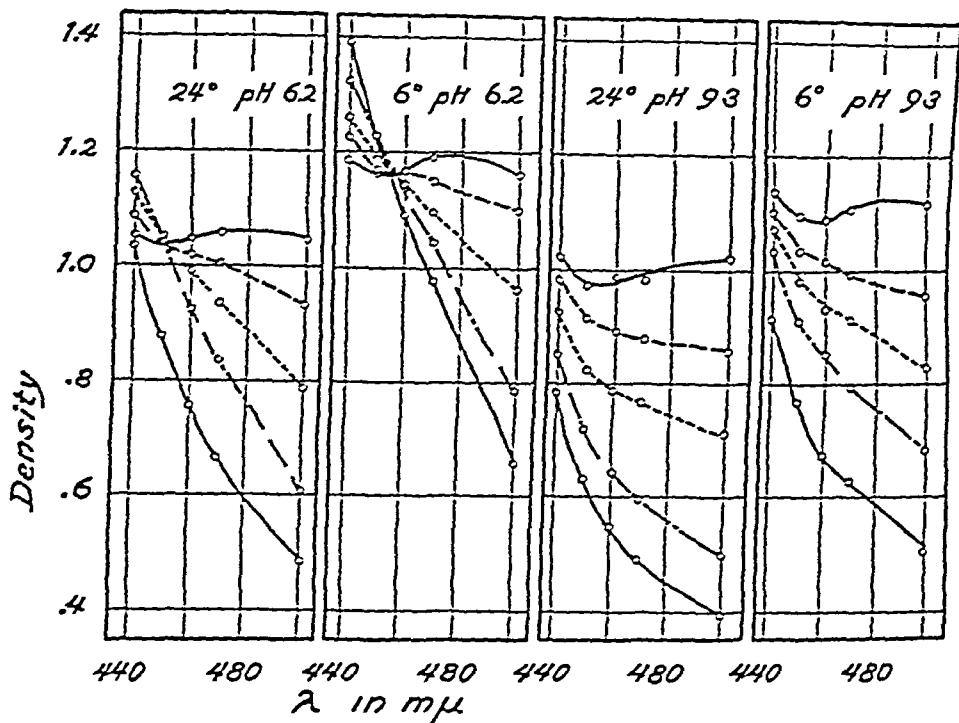


FIG 7. Absorption spectra of four samples of a visual purple extraction, measured at various stages of bleaching at 6° and 24°C in pH 6.2 and 9.3 buffer. The complexities which occur during illumination in acid are greatly increased at low temperature. The bleaching of the alkaline samples, on the other hand, is not affected by temperature. See Table IV. The density values plotted are those of the whole solution.

tochemical reaction, temperature should affect its rate. To test this, four samples of a winter visual purple extraction were placed in small test tubes and two buffered at pH 9.3 while the others were buffered at pH 6.2. An acid and basic sample were bleached at 24°C and the absorption spectrum was measured at various stages during the

bleaching. The other two samples were illuminated and measured at 6°C.³

TABLE IV

Effect of Temperature during Illumination of Visual Purple Buffered at pH 6.2 and 9.3. Each Sample Contains 1.0 Ml of Visual Purple Solution and 0.5 Ml of Buffer Solution

Temperature	pH	λ in m μ	Density after various times of illumination in seconds				
			0	7	20	60	600
C	6.2	440	1.187	1.226	1.260	1.323	1.391
		450	1.162	1.168	1.187	1.226	1.226
		460	1.168	1.168	1.144	1.138	1.091
		470	1.194	1.150	1.097	1.046	0.976
		500	1.163	1.102	0.961	0.787	0.659
24.0	6.2	440	1.051	1.085	1.126	1.162	1.035
		450	1.040	1.040	1.046	1.040	0.878
		460	1.046	1.019	0.992	0.926	0.757
		470	1.057	1.003	0.936	0.836	0.667
		500	1.046	0.931	0.787	0.608	0.484
6.0	9.3	440	1.138	1.102	1.074	1.035	0.916
		450	1.097	1.035	0.982	0.911	0.770
		460	1.085	1.013	0.931	0.850	0.675
		470	1.108	0.992	0.911	0.796	0.628
		500	1.114	0.951	0.827	0.683	0.502
24.0	9.3	440	1.024	0.982	0.926	0.850	0.787
		450	0.971	0.916	0.823	0.719	0.635
		460	0.987	0.892	0.787	0.643	0.549
		470	0.982	0.878	0.766	0.597	0.491
		500	1.019	0.855	0.711	0.498	0.392

The results of this experiment are given in Table IV and Fig. 7. In the acid buffered samples disappearance of the yellow color upon prolonged, intense illumination is markedly retarded at the low as

³ The low temperature was attained by opening windows and shutting off heat, so that since the room temperature was almost the same as that of the solution in the absorption cell, moisture condensation on the glass surfaces was avoided. The use of a water bath with evacuated double walls or of some similar device, was thus unnecessary. The bleaching and measurements at 24°C. of course involved no difficulties.

compared with the high temperature. The alkaline-buffered samples, on the other hand, are unaffected by temperature, indicating that limiting chemical reactions are absent under these conditions. The slight differences in absolute initial density of the four samples are probably due to error in measuring the volumes of solutions used.

This experiment raises the possibility that the difference in behavior of summer and winter visual purple extractions, in regard to appearance of yellow color during illumination, may be partly due to temperature differences at the time of measurement. The room temperatures during the summer months ran as high as 28°C , while in the winter months measurements were often made at temperatures of 18°C . Measured at the lower temperature, the yellow color should be more pronounced than at the higher temperature since its disappearance would be retarded.

Hosoya (1933) has reported that the yellow color resulting from the partial bleaching of visual purple gradually disappears in the dark, leaving the solution colorless. Wald (personal communication) finds the same thing only in perfectly fresh solutions. We have not encountered this effect until recently. Our own data, although at present only preliminary, indicate that with summer extractions at 28°C fading in the dark after illumination and during measurement may be a factor in producing the typical summer result, since the intermediate yellow color, though originally formed, may escape detection under such conditions.

VII

Kinetics

Hecht (1921) has shown by matching colorimetrically against standard mixtures of bleached and unbleached visual purple solutions that the disappearance of visual purple upon illumination obeys a first order equation. The extractions showed no formation of yellow color during intermediate stages of bleaching.

Spectrophotometric measurements of summer extractions confirm these findings. Two experiments are shown in Fig 8. If the kinetics are first order, a straight line should describe the data when logarithm of density is plotted against time of illumination. This is the case

Fig 8 also contains similar measurements of visual purple from winter frogs, which cannot be fitted by a straight line when similarly plotted. In these and subsequent kinetic measurements the density was determined at 500 m μ where the density changes most and visibility is good.

Kinetics measurements in presence of acid and basic buffer behave like winter and summer solutions respectively. A sample of a winter extraction buffered at pH 9.3 conforms to a first order equation over 90 per cent of the course of the bleaching reaction, whereas another

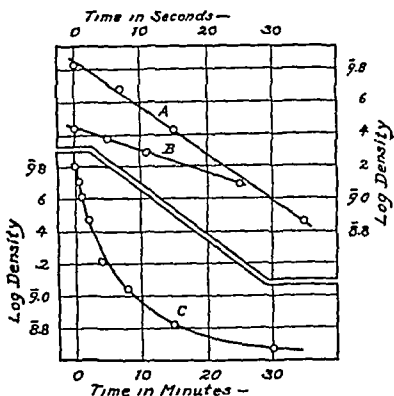


FIG 8 Bleaching kinetics of unbuffered summer and winter visual purple extractions. A form of the first order equation describes the kinetics of the two summer extractions, A and B, but not those of the winter extraction C.

sample of the same extraction buffered at pH 6.2 is more nearly describable by a second order equation. At pH 7.3 neither equation fits the data. Representative data of kinetics measurements at pH 9.3 and 6.2 are given in Table V and plotted in Fig 9.

The absolute density of the whole solution is plotted as originally measured, and the theoretical curves are calculated in the same terms. The first order equation

$$k = \frac{1}{t} \ln \frac{(d - d_{\infty})}{(d_1 - d_{\infty})}$$

where d_0 is the initial density of the unilluminated solution, d_∞ of the fully bleached solution, d_t is the density at time t of illumination, and $k = 0.146$, describes the pH 9.3 data. The value of d_t can be calculated by putting the equation into the form

$$d_t = \frac{[(\text{antiln } kt) d_\infty] + d_0 - d_\infty}{(\text{antiln } kt)}$$

TABLE V

Bleaching Kinetics of Two Samples of a Winter Visual Purple Extraction Buffered at pH 9.3 and 6.2 Respectively and Measured at 500 mμ. The Values of Density Observed Are for the Whole Solution, Exactly As Measured

Time of illumination	Density observed	
	Buffered at pH 9.3	Buffered at pH 6.2
<i>min</i>		
0.0	0.911	0.878
0.5	0.869	0.836
1.0	0.827	0.800
1.5	0.779	0.770
2.0	0.749	0.749
3.0	0.695	0.691
4.0	0.635	—
5.0	0.586	0.628
7.5	0.495	0.578
10.0	0.436	0.534
12.5	0.395	0.513
15.0	0.372	0.495
20.0	0.342	0.467
25.0	0.326	0.453
30.0	0.313	—
34.0	—	0.436
40.0	0.306	—
45.0	—	0.419
63.5	—	0.405
Fully bleached	0.287	0.382

Curiously, the pH 6.2 data are very nearly described by a second order reaction kinetics. The equation used for computing is

$$d_t = \frac{d_0 - kt(d_\infty - d_0 d_\infty)}{1 + kt(d_0 - d_\infty)}$$

where $k = 0.447$

The alkaline sample bleaches much faster than the acid one. The initial difference of 0.034 in density is accidental and is due to error in measuring the small volumes. After 15 minutes of illumination the two curves have crossed one another, and the absolute density value of the alkaline sample is then 0.123 less than that of the acid one.

The apparent second order kinetics of winter and of acid solutions are probably due to the yellow color formed during bleaching, which

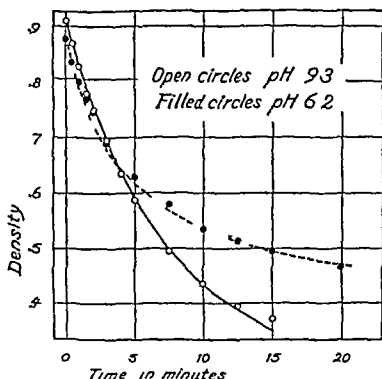


FIG. 9. Bleaching kinetics of two samples of a winter visual purple extraction buffered at pH 9.3 and 6.2 respectively. The solid line is the theoretical curve calculated in terms of a first order equation. The broken line is the theoretical curve calculated according to a second order equation. The apparent conformity of acid visual purple solutions to second order kinetics is probably fortuitous. See Table V.

contributes progressively to the measured density, and decreases the apparent speed of bleaching. Another interfering factor under these conditions is the density of the fully bleached solution, which is essential in calculating kinetics. With summer and alkaline extractions, the final stage of bleaching is quite definite. However, in the case of the winter and acid extractions, choice of the final density value is uncertain, due to the appearance of the yellow color. It is necessary

to illuminate for 20 minutes with a photoflood lamp 6 inches above an ice water bath containing the cell in order to reduce the solution to a photo-stable condition. Moreover, the effect of temperature on the disappearance of the yellow color further complicates the situation. Therefore the real kinetics of the disappearance of visual purple are given by the summer and alkaline solutions.

VIII

General Considerations

Kuhne (1879) and Garten (1907) have assumed the yellow color to represent a decomposition product of visual purple, and Garten has even postulated a scheme wherein this intermediate yellow substance is stoichiometrically involved in the regeneration as well as the break-down of visual purple.

A necessary requirement of such a system is that the amount of yellow substance produced be proportional to the visual purple originally present in any extraction. The data make such an interpretation difficult since extractions possessing equal densities of visual purple but made and measured at different seasons of the year show upon illumination all gradations in density of the intermediate yellow color, ranging from zero to a high value.

The possibility that a second light-sensitive substance, present under certain conditions, might be the precursor of the yellow color, is unlikely since no such substance has been found in retinal extracts containing visual purple, although some other light-sensitive substance peculiar to the cones may be present in concentrations too minute for measurement by the present methods.⁴

⁴ von Studnitz (1932), working with turtle retinas which contain only cones, reported the presence of a light-sensitive substance which could not be extracted as is rod visual purple, but which underwent a decrease in density in the retina upon exposure to light. The density changes which he reported are very small and are complicated by other density changes in the opposite direction. Also, it is quite likely that pigment migration and other photo-mechanical effects contributed to the values which he obtained. Factors of this sort would have a marked influence on measurements of light transmission through a semi-opaque tissue like the retina, where dispersion is pronounced. The values which he gives for the absorption spectrum of this material seem quite meaningless since the

If conclusions drawn from experiments on the whole retina are applicable to retinal extractions, it is possible that a part of the intermediate yellow color may be due to retinene (Wald, 1934), and if this be true the seasonal variation may in part involve a fluctuation in vitamin A. Wald (1933) has demonstrated the presence of this vitamin in the frog's retina, and Fridericia and Holm (1925), later confirmed by Tansley (1931), have shown the regeneration of visual purple in the white rat to be dependent upon vitamin A. Its effect upon human dim vision is well known (Medical Research Council, 1932).

I wish to express my gratitude to Dr. Selig Hecht, who suggested this problem and who has helped me, both in devising certain of the experiments, and in the subsequent treatment of the data. I also wish to thank Mr. Simon Shlaer, of the Biophysics Laboratory, for planning and constructing the optical system used in connection with the spectrophotometer lamp and for numerous ingenious practical suggestions.

SUMMARY

Visual purple from winter frogs shows an intermediate yellow color during bleaching by light, summer extractions do not. This seasonal effect can be duplicated by variations in the hydrogen ion concentration and in the temperature of the solutions. Increasing the pH approximates the summer condition, while decreasing the pH approximates the winter condition. Temperature has no effect on the bleaching of alkaline solutions but greatly influences acid solutions. At low temperatures the bleaching of acid solutions resembles the winter condition, while at higher temperatures it resembles the summer condition.

A photic decomposition product of frog retinal extractions is an acid base indicator: it is yellow in acid and colorless in alkaline solution. Its color is not dependent upon light.

The hydrogen ion concentration of visual purple solutions does not change under illumination, nor is there a difference in the pH of sum-

color of the whole retina was considered instead of that of the light sensitive substances alone. Also, his location of a maximum of absorption even for the retina as a whole, at 560 $m\mu$, seems unwarranted upon critical examination of his data.

mer and winter extractions Bile salt extractions of visual purple are usually slightly acid

The conflicting results of past workers regarding the appearance of "visual yellow" may be due to seasonal variation with its differences in temperature, or to the presence of base in the extractions It is also possible that vitamin A may be a factor in the seasonal variation

The photic decomposition of visual purple in bile salts solution, extracted from summer frogs, follows the kinetics of a first order reaction Visual purple from winter frogs does not conform to first order kinetics Photic decomposition of alkaline, winter visual purple extractions also follows a first order equation Acid, winter extractions appear to conform to a second order equation, but this is probably an artefact due to interference by the intermediate yellow

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THE ELECTRICAL CHARGE OF MAMMALIAN RED BLOOD CELLS

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INTRODUCTION

Although the electrophoretic mobility of mammalian erythrocytes has been investigated to some extent, suitable estimation of their surface electrical charge has not as yet been presented. Recent developments in the theories of electrokinetic phenomena and in the theories of dilute solutions of electrolytes justify the calculation of the density of net surface charge from the available quantitative data on the electric mobility of red cells. In addition, through the work of Ponder, measurements of the surface areas of red cells permit a calculation of the effective net charge per red cell. The results of these calculations now show that the differences in electrokinetic potential (which is directly proportional to the electric mobility) calculated for the blood cells of a series of mammals bear no simple relationship to the net charge for the cells of each member of the series. These calculations thus throw new light on the differences in the physicochemical nature of the red cell under the experimental conditions employed and suggest further experimentation.

Theoretical

Since the electric mobility of red cells in salt solutions is independent of their orientation in the electric field (1) and since erythrocytes made spherical by traces of saponin have electric mobilities, within the limits of error, identical with the disc shaped cells suspended in the same buffer, the electric mobilities of these microscopic particles can be treated by the theory derived for large particles (2). We can,

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with von Smoluchowski, calculate the ζ -potential from the electric mobility, v ,¹

$$\zeta_E = \frac{4\pi\eta}{D} v, \quad (1)$$

(all units centimeter-gram-second and electrostatic units of charge), assuming that the viscosity, η , and dielectric constant, D , in the diffuse double layer do not assume values which are very different from those of the medium.² In any event, the measurements of v were carried out in the same medium so that any future corrections in the values of these constants would probably only change our results by a proportionality factor. From ζ_E , the net charge density, σ , on a surface may be calculated (2, 5, 6) in solutions containing any number of positive ions of the type, z , and negative ions of the type, j , by means of the generalized theory of Gouy, valid for large particles,

$$\sigma = \sqrt{\frac{NDkT}{2000\pi}} \sqrt{\sum c_i \left(e^{-z_i \frac{e\zeta}{kT}} - 1 \right) + \sum c_j \left(e^{+z_j \frac{e\zeta}{kT}} - 1 \right)}, \quad (2)$$

where N is Avogadro's number, k , the Boltzmann constant, e , the electronic charge, z , the valence, T , the absolute temperature, and c , the ionic concentrations in mols per liter existing in the body of the solution. σ has the same sign as ζ , all units are in centimeter-gram-second and electrostatic units of charge.

Inspection of this equation reveals that σ , under our conditions,

¹ In general, it is desirable to calculate the ζ -potential rather than combine equations (1) and (2) (*vide infra*) because of the possible dependence of the electric mobility on the radius under other than the present conditions.

² "This assumption is not altogether unjustified for the following reasons

1 Substitution of D and η of the solvent in the Onsager (3) conductance theory yields for concentrations up to about 0.05 N (simple salt solutions) satisfactory values for the limiting slopes and changes in mobility with concentration.

2 If the electric mobility of microscopically visible quartz particles covered with a film of adsorbed protein is studied in different concentrations of alcohol (4), it is possible to correlate the surface potential and surface charge calculated from these mobilities with the charge obtained by another (thermodynamic) method. As far as these results go, the characterization by the two parameters, viscosity and dielectric constant of the solvent, in the Helmholtz-Debye theory is correct within 10 per cent" (2).

will depend only on ζ , for all of the other terms are constants in a given solution of electrolyte. Simplifying equation (2) by collecting constants (except those here given by the concentration and valence) there is obtained

$$\sigma = \alpha \sqrt{\sum c_i \left(e^{\frac{-z_i \zeta}{\beta}} - 1 \right) + \sum c_j \left(e^{\frac{+j \zeta}{\beta}} - 1 \right)}, \quad (3)$$

where $\alpha = 17,600$ and $\beta = 0.0256$ volt at 25°C . If ζ in volts is introduced into this equation with proper regard to its sign, the resultant value for σ will be in electrostatic units of charge. Since σ is the net charge per square centimeter, the effective net charge per cell may be calculated if the surface area of the cell is known.

RESULTS

The values of the charge were calculated by equation (3) from data obtained with various mammalian red cells (1) in isotonic ($M/15$) phosphate buffers at pH 7.4. In this case, the problem is complicated by the presence of three ionic types³ a single positive univalent type, i , and two negative types, j (H_2PO_4^-) and jj (HPO_4^{--}), of different valences. The method of calculation is illustrated as follows.

Let $v = -1.00 \mu\text{sec/volt/cm}$, then $\zeta = -0.0128$ volt, $z = 1$, $z_i = 2$, $z_{jj} = 1$, and $c_i = 0.120$, $c_j = 0.0133$, $c_{jj} = 0.0533$, so that,

$$\sigma = 17,600 \sqrt{0.120 \left(e^{\frac{-1 \times (-0.0128)}{0.0256}} - 1 \right) + 0.0533 \left(e^{\frac{+2 \times (-0.0128)}{0.0256}} - 1 \right) + 0.0133 \left(e^{\frac{+1 \times (-0.0128)}{0.0256}} - 1 \right)}$$

Through the kindness of Ponder, we have been furnished with values for the surface areas (found by methods described in detail in his monograph (7)) of the various red cells investigated, with the exception of those for the sloth, where no data were available. It will be noted (Table I) that the net charge of the red cell does not vary in the same order, from species to species, as σ , the charge per unit area. Nor does there seem to be any clear relationship between net charge per cell and zoological classification.

By dividing the net charge by the electronic charge (4.77×10^{-10} e.s.u.), the number of effective electrons at the surface has been calcu-

³ The concentrations of the H^+ and PO_4^{--} ions are here neglected

lated (Table I, Column 7) For example, in the case of man, there are fifteen million electrons on each red cell, the highest value among these mammals One might say that this corresponds to the "valence" of each cell A similar computation of the net charge has been made for the typhoid bacillus (8) By assuming that each effective electronic charge occupies an ionic area of, say, $1 \times 10^{-15} \text{ cm}^2$, the percentage of the surface occupied by these charges may be roughly estimated (Table I, Column 8) The values never rise far above 1 per cent, which agrees in magnitude with data obtained on other surfaces (2)

TABLE I

Animal	Mobility	ξ	σ	Area	Net charge	Number of electrons	Area occupied
	μ/sec	volts	$e s u$	$\text{cm}^2 \times 10^3$	$e s u \times 10^3$	$\times 10^{-6}$	per cent
Rabbit	0 55	0 00704	1890	1 10	2 08	4 37	0 40
Sloth	0 97	0 0124	3330	—	—	—	—
Pig	0 98	0 0125	3360	0 95	3 19	6 70	0 70
Opossum	1 07	0 0137	3680	1 56	5 74	12 0	0 77
Guinea pig	1 11	0 0142	3780	1 15	4 35	9 14	0 80
Man	1 31	0 0168	4500	1 63	7 34	15 4	0 94
<i>Rhesus</i> monkey	1 33	0 0170	4570	1 37	6 26	13 2	0 96
Cat	1 39	0 0178	4780	0 80	3 82	8 03	1 00
Mouse	1 40	0 0179	4800	0 96	4 61	9 70	1 01
Rat	1 45	0 0186	4980	1 02	5 08	10 7	1 05
Dog	1 65	0 0211	5660	1 22	6 90	14 5	1 19

DISCUSSION

In general, for small values of v , the Debye approximation,

$$\sigma = \frac{D}{4\pi} \xi \kappa, \quad (4)$$

may be employed It was not clear to the writers whether this equation would give values in M/15 phosphate buffer different from those obtained with equation (3) In Fig 1 are plotted $\sigma - v$ curves for this phosphate buffer, calculated both by means of the exact formula (Equation 2) and by the approximate formula given by equation (4) Note that in the range of v (up to 1.65μ per sec) encountered in this

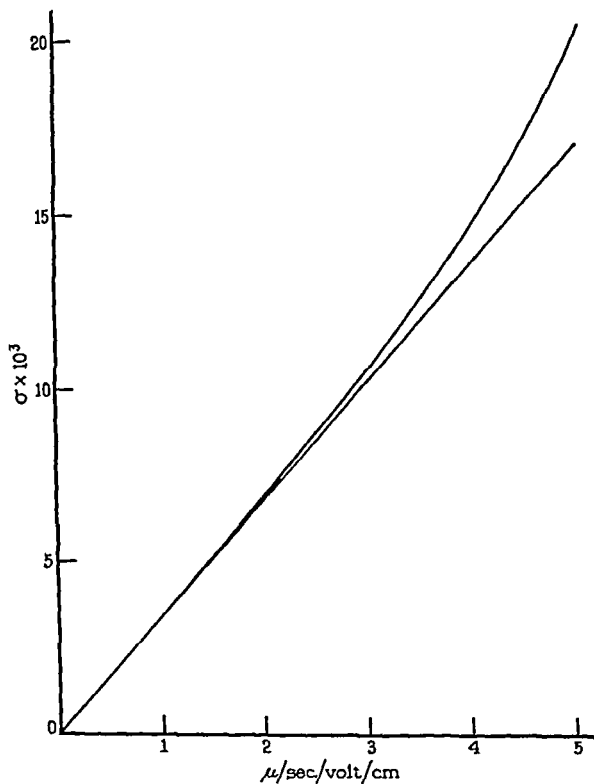


FIG 1 The straight line has been calculated according to the Debye approximation and the curved one by means of equation (2) for $M/15$ phosphate buffer

investigation, values of σ calculated by the two methods agree within the limits of error. Important divergences occur above 2μ per sec

The changes in the surface chemistry of the red cell due to alteration of the suspending medium may be investigated with profit by means of the method utilized to calculate the charge. Thus, mammalian red cells increase their electric mobilities in isotonic glucose buffered slightly by phosphate (1). Advantage could be taken of this effect to determine if the net charge is affected or if it is only the ζ -potential which varies. Specific ion or molecular effects could be more closely followed. The same procedure is, of course, applicable to other types of cells.

In a short series of experiments on the electrophoretic mobilities of red cells in twelve cases of varying types of anemia (1), it was found that both the macrocytes and microcytes when suspended in the same phosphate buffer have mobilities, with few exceptions, which are identical, within the limits of error, with the mobility of erythrocytes from a normal individual. Theory demands that large particles which exhibit identical mobilities in solutions of the same ionic concentration must in each case have an equal number of charges per unit area (2). Obviously if σ is nearly the same for both normal cells and the cells of abnormal size found in the anemias, the net charge per cell must be markedly different, for the two types of cells have very different surface areas. Hence some mechanism seems to exist, capable of stabilizing the charge per unit area, within limits, while the surface undergoes comparatively marked changes in area and shape. The conditions which must be satisfied to establish the identity of two surfaces have been discussed before (9).

SUMMARY

From data on the surface area and electrical mobilities of mammalian red blood cells in $M/15$ phosphate buffer at pH 7.4, it has been possible, with the help of the Gouy and von Smoluchowski theories, to calculate the net surface charge per cell as well as the charge per unit area. It was found that a single mammalian red cell has a net surface charge ranging from four to fifteen million electrons, depending on the species. No clear relationship between zoological classification and surface charge is apparent. It is suggested that a mechanism exists which is capable of keeping the surface density of net charge constant when comparatively large changes in surface area occur in the anemias.

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ELECTRIC IMPEDANCE OF ASTERIAS EGGS

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Several years ago the absolute value of the alternating current impedance of suspensions of *Arbacia* eggs was measured at frequencies from 1 to 15,000 kc (Cole, 1928*b*). It was concluded that the egg membrane capacity was not as nearly a static capacity as the capacity of the red blood cell membrane (Fricke, 1925*a*, 1933), but was of the polarization impedance type, similar to that which could be attributed to the cell membranes of tissues (Cole, 1932, 1933, Bozler and Cole, 1935). Recent measurements (Cole, 1935) of both the resistance and reactance components of suspensions of *Hipponoe* eggs at frequencies from 11 to 2,300 kc showed the membranes to have static capacities and suggested complicating phenomena at the upper end of the frequency range. It was then obvious that more complete measurements should be made not only on *Arbacia* eggs, but also on the eggs of other echinoderms.

Material

The availability and the large size of the eggs of the common starfish *Asterias forbesi* made them good material for this work. As soon as the animals were collected the ovaries were removed and placed in sea water until the eggs had been shed. The eggs were washed once or twice, centrifuged lightly, and drawn into the conductivity cell. The suspension of eggs separated by jelly reached a constant resistance in 15 or 20 minutes which was maintained for an hour or two. When the suspension was removed from the cell it was found that very little cytolysis had taken place. A singular scarcity of ripe males prevented tests of viability and measurements of fertilized eggs. All measurements were made between 21 and 22°C.

Apparatus

The conductivity cell was of the burette type used for the *Hipponoë* eggs. It has a volume of 1.51 cc and a cell constant of 14.53. The electrodes were platinized platinum.

The measurements of the suspensions were made with the conductivity equipment of the Biophysics Laboratory at Cold Spring Harbor which was placed at our disposal by Dr. Hugo Fricke. Substitution measurements of the parallel resistance and capacity were made with the Wheatstone bridge from 1 to 2048 kc and with the resonance circuit from 4.1 to 16.4 megacycles.

An electrolytic resistor of the type employed in the work on the sartorius muscle (Bozler and Cole, 1935) and the *Hipponoë* eggs (Cole, 1935), was used

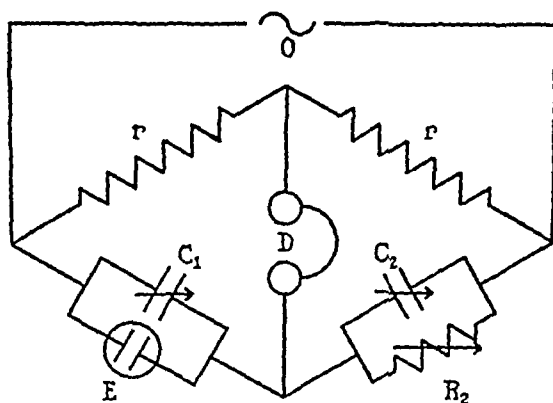


FIG. 1. Wheatstone bridge with egg cell (E), calibrated condensers C_1 and C_2 and variable resistance R_2 (r and r are fixed equal resistances), oscillator (O), and detector (D).

throughout as a variable standard of resistance. It is a modification of the micrometer electrolytic cells described by Miller, 1923, Fricke and Curtis, 1935 *b*, and Jones and Christian, 1935.

At each frequency, the bridge, Fig. 1, was balanced with the condenser C_1 set to minimum capacity. The electrolytic resistor (R_1) was then substituted for the egg conductivity cell (E) and, leaving R_2 and C_2 unchanged, the bridge was balanced by adjusting R_1 and C_1 . The resistance of the suspension was then known from the calibration of the resistor at low frequency. The capacity due to the eggs was

$$C = \Delta C + C_R - C' + \frac{\Delta L}{R^2},$$

where ΔC is the change in capacity of the parallel condenser (C_1) on substitution,

C_R is the capacity of the electrolytic resistor, C' is due to the capacity of the egg cell filled with sea water and the change in capacity of the connecting wires on substitution, and $\Delta L/R^2$ is the capacity correction due to a difference ΔL in the inductance of the leads when the circuit resistance is R . On the assumption that C_R is due to water alone, it is found that

$$C_R = \frac{71}{K} \mu\mu f$$

where K is the cell constant (Tricke and Curtis, 1935*b*). For the electrolytic resistor $K = K_1 M$ where K_1 is the cell constant for 1 cm electrode separation and M is the micrometer reading in centimeters so

$$C_R = \frac{71}{K_1 M} \mu\mu f$$

If it is assumed that ΔL is negligible and that the conductivity cell is filled with electrolyte, then

$$\Delta C = \frac{71}{K_1 M} - C$$

When the conductivity cell is filled with electrolytes of different conductivities so that M varies ΔC is a linear function of $1/M$. These data for both the bridge and the resonant circuit are plotted in Fig. 2. For the former, $C' = 2.87 \mu\mu f$, and for the latter $C' = 1.05 \mu\mu f$.

The cell constant $K_1 = 0.907$, so the slopes of the lines should both be 7.83. Actually they are found to be 8.36, so that probably the glass wall of the cell and the surrounding air contribute to the capacity to this extent. The latter value is used to compute C_R . The graph further shows that ΔL is negligible.

The electrodes of the conductivity cell and the electrolytic resistor were platinized so that, with sea water in the former, the equivalent parallel capacity of each and their difference was small but it was still necessary to correct for the electrode polarization capacity at low frequencies. If C_W is the change in parallel capacity when the micrometer cell is substituted for the conductivity cell filled with sea water, and C is the difference in capacity when the cell is filled with suspension, then the capacity due to the eggs

$$C_E = C - \left(\frac{R_W}{R} \right)^2 C_W$$

where R_W and R are the parallel resistances of the sea water and the suspension respectively.

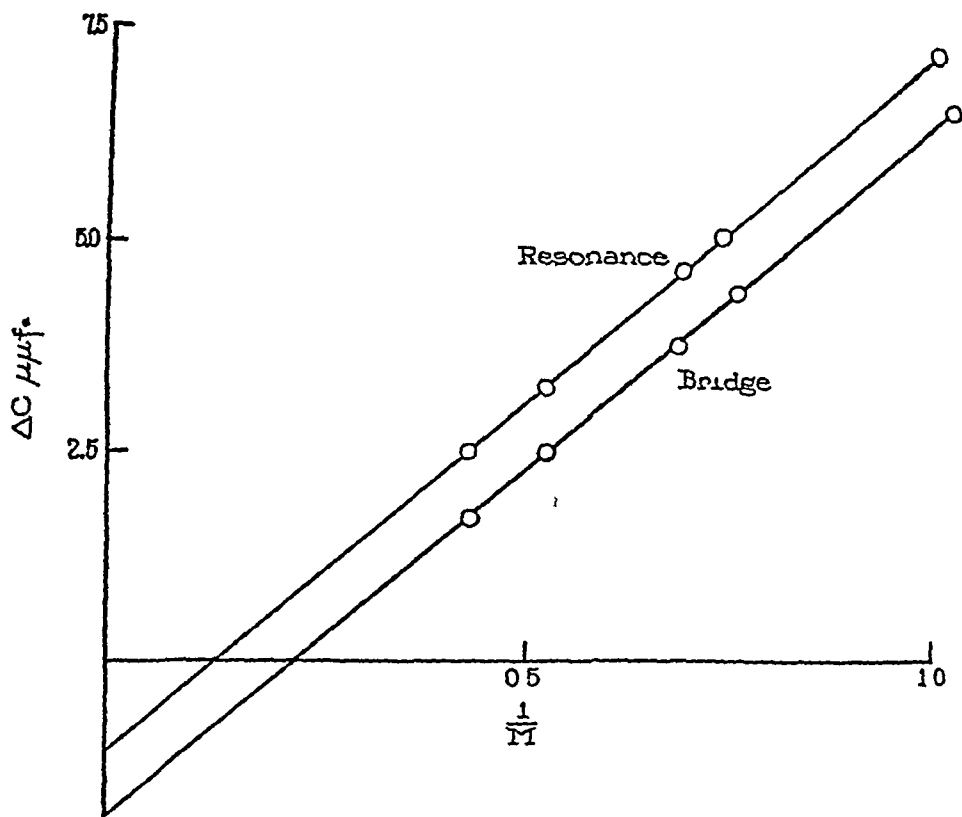


FIG 2 Reciprocal of electrolytic resistor setting, $1/M$, vs capacity difference, ΔC , between resistor and conductivity cell

Data and Calculations

When a suspension of eggs is equivalent to a circuit containing two resistances and a single static capacity, the complex locus (Cole, 1928a, 1933) will be a semicircle with its center on the resistance axis. The complex plane impedance locus is obtained by plotting the series resistance R_s and the series reactance X_s , as abscissae and ordinates. These should be calculated from the parallel resistance R and capacity C_E by the formulae

$$R_s = \frac{R}{1 + R^2 C_E^2 \omega^2}, \quad X_s = \frac{R^2 C_E \omega}{1 + R^2 C_E^2 \omega^2},$$

where $\omega = 2\pi n$ and n is the frequency. In almost every case, however, the term $R^2 C_E^2 \omega^2$ was so small¹ that it was neglected. Then

¹ In Table I, $R^2 C_E^2 \omega^2$ has its maximum value in No. 8 at 128 kc and here $R^2 C_E^2 \omega^2 = [930.5 \times 117.2 \times 10^{-12} \times 2\pi \times 128 \times 10^3]^2 = 7.7 \times 10^{-3}$

TABLE I

Suspension of Unfertilized Asterias Eggs of Diameter 124 μ

Resistance of sea water, $R_W = 450.0$ ohms Volume concentration $\rho = 47.1$ per cent
 Extrapolated capacity, $C_0 = 335$ μf Cell constant, $K_2 = 14.53$
 Temperature, 21.8°C

Frequency		R and R_S	C_E	X_S
No	n			
		ohms	μf	ohms
1	1 10^3	1054.9	380.2	2.78
2	2	1054.7	360.6	5.13
3	4	1054.0	349.4	9.84
4	8	1052.5	337.7	21.39
5	1.6 10^4	1047.0	323.6	37.65
6	3.2	1029.3	290.5	65.15
7	6.4	986.0	222.3	87.05
8	1.28 10^4	930.5	117.2	86.05
9	2.56	890.5	47.98	61.10
10	5.12	872.6	15.98	39.05
11	1.024 10^4	860.0	5.86	27.86
12	2.048	854.5	2.88	27.00
13	4.1	848.5	2.06	38.12
14	8.2	834.0	1.38	49.45
15	1.64 10^7	810.0	0.73	48.95

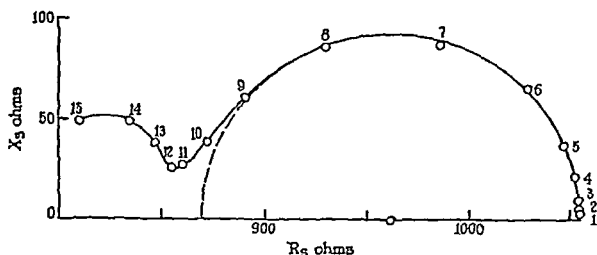


FIG 3 Complex plane locus, series resistance R_S , vs series reactance X_S for a suspension of unfertilized *Asterias* eggs. Frequencies given in Table I

$R_S = R$ and $X_S = R^*C_E\omega$. It is seen from the data for an *Asterias* suspension given in Table I and plotted in Fig 3 that such a circuit is a close approximation over the low frequency range. It should then

also be true that at sufficiently low frequencies the parallel capacity should be constant and independent of frequency. This is, however, not the case, for in Table I and Fig 4 the parallel capacity continues to increase as the frequency decreases without the corresponding in-

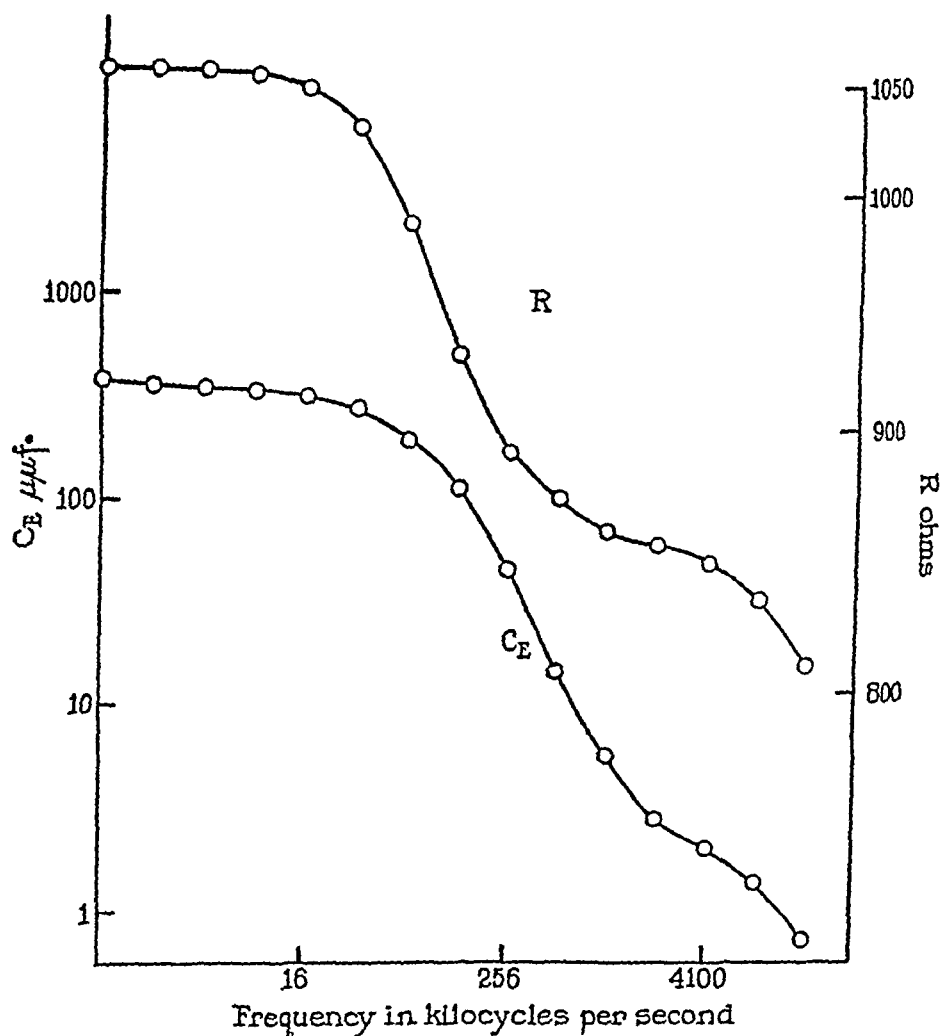


FIG 4 Log frequency, n , vs log parallel capacity, C_E , and log parallel resistance, R , for a suspension of unfertilized *Asterias* eggs

crease in resistance that would be expected if the current was still penetrating the egg interior to any appreciable extent. It has been shown that a random distribution of spheres having a uniform conducting interior and a poorly conducting membrane of capacity C_M

per unit area in a suspending medium is equivalent to the network A of Fig. 5 (Fricke and Morse, 1925, Fricke, 1933). It is then found that (Cole, 1928 *a*)

$$C_M = \frac{2 C_0 K_s}{\left(2 + \frac{R_W}{R_0}\right) \left(1 - \frac{R_W}{R_0}\right) a}$$

where a is the egg radius, K_s the conductivity cell constant, R_W and R_0 the resistances of sea water and the suspension, while C_0 is

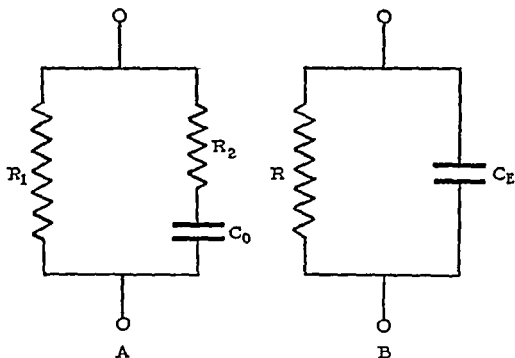


FIG. 5. Equivalent circuits.

the low frequency capacity. The problem now is to determine the C_0 which is the dominant constant over the intermediate frequency range by an extrapolation to low frequency and thus eliminate the confusing factors at the low frequencies which prevent the parallel capacity (C_E) from becoming constant. When the suspension is represented by Circuit A in Fig. 5, the equivalent parallel resistance (R) and capacity (C_E) at any frequency are given by

$$\frac{1}{R} = \frac{1}{R_1} + \frac{\omega^2 R_2 C_0}{1 + \omega^2 R_2^2 C_0^2}, \quad C_E = \frac{C_0}{1 + \omega^2 R_2^2 C_0^2},$$

from which

$$\frac{1}{R} = \frac{1}{R_L} - \left(\frac{1}{R_L} - \frac{1}{R_0} \right) \frac{C_E}{C_0} \quad (1)$$

When C_E is plotted against $1/R$ a straight line should result for the range in which the above assumptions are valid. The intercept on the $1/R$ axis should correspond to the extrapolated value at the high frequency end of the circle, and this may be called $1/R_L$. At the other

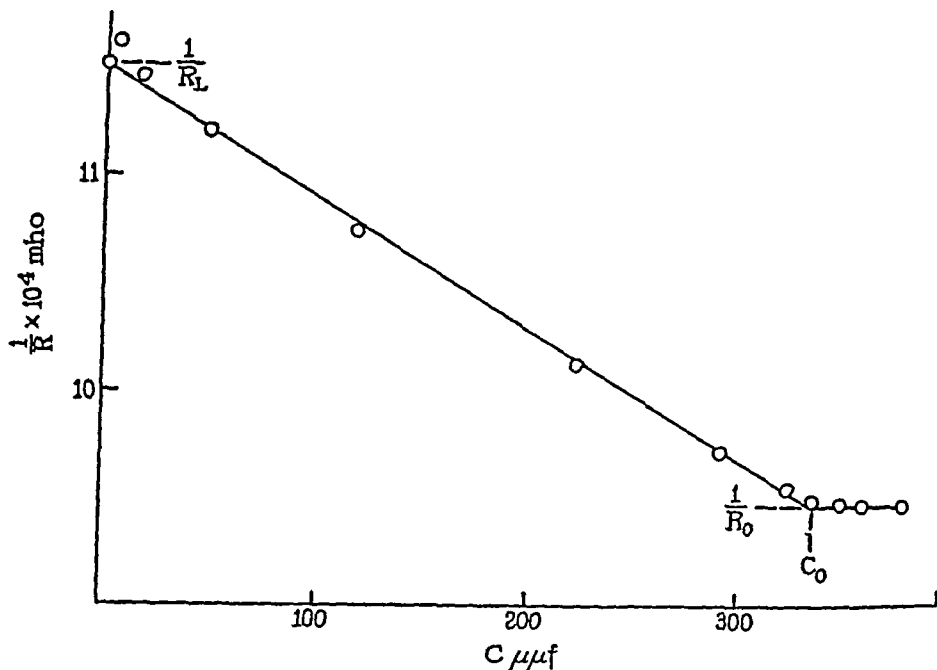


FIG 6 Parallel capacity, C_E , vs reciprocal resistance, $1/R$, for *Asterias* suspension. $C_0 = 335 \mu\mu f$

end where $1/R = 1/R_0$ we have $C_0 = C_E$, as shown in Fig 6. Here $C_E = 335 \mu\mu f$, $R_W/R_0 = 0.421$, $a = 62 \mu$, so $C_M = 1.03 \mu f/cm^2$. The average value for unfertilized *Asterias* eggs is $1.10 \mu f/cm^2$. Two runs taken on a single batch of eggs at different volume concentrations gave values of C_M which differed by less than 2 per cent. It is obvious from the failure of the low frequency data to follow equation (1) that the capacity which determines the penetration of current into the egg is only part of the capacity which is observed at low frequencies. Any resistance component accompanying this additional capacity was not observable.

Ignoring for the moment the complicating factors at high frequencies, we may assume that the extrapolated value of resistance R_L at the high frequency end of the semicircle represents the equivalent low frequency internal resistance which the egg would have if it were uniform. In the data represented by Fig. 3, we find $R_L/R_W = 6.93$, while the average value of all runs gives $R_L/R_W = 7.24$. For the unfertilized *Hipponoe* egg $R_L/R_W = 11$. Even before the abrupt entrance of the high frequency element there is a general tendency for the data to depart from the circle slightly. This was found to about the same extent for *Arbacia*, but was not so apparent in the *Hipponoe* data. Without data at much higher frequencies, it is hazardous to make any analysis of the high frequency effect or assign its cause to any particular part of the egg structure. Making a most unjustifiable extrapolation to infinite frequency we find that if no further undiscovered elements are present, the average high frequency internal resistance of the egg is about 4.5 times the resistance of sea water.

DISCUSSION

It should be pointed out that essentially we have used an electrolyte as our standard resistance and that any frequency dependent characteristics which electrolytes may have must be considered as possible sources of error. There are two questions which may well be mentioned here, not because they are of particular importance in this specific problem, but because they are so often asked when conductance phenomena at high frequency are discussed.

It is well known that at high frequencies the current density in the interior of a conductor is less than at the surface and that the effective resistance is greater than at low frequencies. An approximate analysis of this "skin effect" shows that for a conductor having a resistance R ohms per centimeter, the increase in resistance ΔR is given by

$$\frac{\Delta R}{R} = \frac{\omega^2}{12 \cdot 10^{12} R^2}$$

For sea water in a cell having a square centimeter cross section, R is about 25 ohms per centimeter and the resistance error will be less than 1 per cent at a frequency of 10^9 cycles per second.

On the other hand, the resistance of an electrolyte is less at high frequencies because the effect of the electrostatic interaction between ions is reduced. For N/100 KCl, approximately the concentration used in the electrolytic resistor, this effect is less than 1 per cent at $2 \cdot 10^8$ cycles per second.

The measurements and analysis indicate that a membrane having a static capacity independent of frequency, of about $1 \cdot 10 \mu\text{f}/\text{cm}^2$, controls the passage of the current through the interior of the *Asterias* egg. This is the same type of capacity and of the same order of magnitude as that found for other suspended cells, as Table II shows.

As is shown in the following paper, the apparent paradox between *Hipponoe* eggs on the one hand and *Arbacia* eggs and tissues on the other has now resolved itself into the reasons for differences between tissues and suspended single cells given by the same analysis and the explanation of the earlier results on *Arbacia* eggs.

It is extraordinarily interesting that cells differing so widely in other characteristics should have membranes so similar in both the type and the magnitude of their electric capacity. All these data naturally lead to the hypothesis of a monomolecular membrane if a dielectric constant of 3 is assumed (Fricke, 1925*a*). It is, however, apparent from the work which has been done on monomolecular films that such a conclusion should be taken as a warning that it is probably necessary to consider the structure and properties of the material in a thin layer rather than in bulk (Danielli, 1935, Danielli and Davson, 1935).

The volume concentration ρ was computed from the low frequency resistance R_0 on the assumption of a non-conducting membrane by the Maxwell formula for a suspension of non-conducting spheres

$$\frac{\rho}{2} = \frac{1 - \frac{R_H}{R_0}}{2 + \frac{R_H}{R_0}}$$

No independent measurements of the volume concentration were made. On the assumption of a membrane resistance of $25 \text{ ohms}/\text{cm}^2$ ($40 \text{ ohms}/\text{cm}^2$ was the order of magnitude for frog sartorius) the resistance of a 50 per cent suspension would be 1 per cent less than if the membrane were non-conducting, whereas the accuracy of volume

concentration determinations is about ± 2 per cent. It should be possible with large amounts of suitable material, adequate temperature control, and sufficient time, to increase the accuracy of the measurements of volume concentrations considerably over those made with *Hipponoe* eggs. On the other hand, several methods are available for measurement of the cell volume concentration in tissues. By such methods it may be possible to determine whether the tissues showing a polarization impedance have a markedly lower low frequency membrane resistance than have the single cells which have been found to have a static capacity. This seems to be the case from the results on frog sartorius muscle and *Hipponoe* eggs, but the data are not sufficiently accurate or numerous to justify a general and definite conclusion. From simple qualitative considerations, it can be seen that when there is selective ion permeability giving rise to a large out-of-phase back electromotive force with a phase angle of less than 90° , there must be a finite membrane conductance due to the passage of all ions to which the membrane is permeable. On this basis it might be said that probably both the total and the selective permeability of the suspended cells is low compared to tissues in general. In this connection it should be noted that the value in radians of the phase angle ϕ (as given by α , where $\alpha = \phi/\frac{\pi}{2}$) for the red cell $\alpha \approx 0.95$ and for the white cell $\alpha = 0.90$ is considerably less than for the three echinoderm eggs (for which α is very close to unity). This would seem to be in line with the relative permeabilities of the red cell and *Arbacia* egg to water.

At present there are not sufficiently accurate data available to warrant an extended discussion of the low frequency capacity effect but we are inclined to the obvious conclusion that it is the same type of phenomenon as that observed for the red cell by Fricke and Curtis (1935a). It was at first felt that this effect might well be due to the selective permeability of the membrane which represented a high impedance as compared with the reactance of the static capacity of the non permeable portions of the membrane.

The experiments of Errera (1923, 1932), Fricke and Curtis (1935c), and others on colloids and the suggestion of Bikerman (1934) lend weight to the view that this is associated with surface conductance

A further support is found in the data of Briggs on cellulose at 1000 cycles where the von Smoluchowski conductance was unexpectedly low (Cole, 1932) Urban, White, and Strassner (1935) also state that the von Smoluchowski conductance of pyrex glass is very slight at 1000 cycles It is not possible, however, to choose between a normal and a tangential conductance explanation of the data on suspended cells

At the higher bridge frequencies, 256 and 512 kc, there is a tendency for the points to lie above the circles which best fit the lower frequency points This is found in the data for unfertilized *Arbacia* eggs, but was present to a lesser degree in the data for unfertilized *Hipponoe* eggs It is not yet certain whether this can be due to the presence of the high frequency element

The intercept of the high frequency end of the circle on the resistance axis was interpreted quite hesitantly for the *Hipponoe* data because it gave so high a value for the internal resistance as compared with the early data for the *Arbacia* egg and those for the red and white blood cell The finding of the high frequency element, which the *Hipponoe* data hinted at, involves the necessity of considering its cause along with the equivalent internal specific resistance It was decided in the work on *Hipponoe* that the cause of the high value for the low frequency internal resistance was not the nucleus If the cytoplasm had the resistance of 3.6 times that of sea water as found for *Arbacia*, the nuclear volume would have to be 60 per cent of the egg volume Since the *Arbacia* figure is now found to be considerably higher and *Hipponoe*, *Asterias*, and *Arbacia* all give about the same value, it is advisable to reconsider the possibility of a nuclear effect, since another element is now known to be present It is interesting to note that from the data on non-conducting lecithinated red blood cells (Fricke, 1933) there is no indication of the entrance of a similar high frequency element, although, if present, it might be expected to have at least a slight effect at the highest frequency

If we consider the nucleus to have a very low internal resistance, a volume of 2 per cent of the egg volume is estimated The effect on the value of the specific resistance of the cytoplasm is then relatively slight and we arrive at an average value of 217 ohm cm, or about 7 times that of sea water

On the other hand, if the nucleus is assumed to have the same resistance as the cytoplasm, the figure is 5 per cent and the internal specific resistance becomes 136 ohm cm or 4.5 times sea water. This is in marked contrast to the red and white blood cells and sartorius muscle when considered in relation to their normal environment, but it is interesting to notice that the absolute values of the internal resistance are not so widely divergent, as is shown in Table II.

The acceptance of so high a value of the cytoplasmic resistance would necessitate the presence of a large amount of undissociated material in order to maintain osmotic equilibrium with sea water and would indicate that in different cells the variations in the osmotic

TABLE II

Suspended cell	C_M $\mu\text{f}/\text{cm}^2$	Internal resistance		Reference
			ohm cm	
Red blood cell	0.95	2 × plasma	140	Fricke and Curtis 1934a
White blood cell	1.0	2 × plasma	140	Fricke and Curtis 1935a
Yeast	0.60	—	—	Fricke and Curtis 1934b
<i>Hippomane</i>				Cole 1935
Unfertilized	0.87	11 × sea water	203	This paper
Fertilized	2.18	(18 × sea water)	(349)	
<i>Asterias</i>				Cole and Cole 1936
Unfertilized	1.10	4-7 × sea water	136-225	
<i>Arbacia</i>				
Unfertilized	0.72	4-6 × sea water	120-186	
Fertilized	3.10	(11 × sea water)	(336)	

pressure of the media are met to a large extent by changes in concentration of non ionized substances.

The volume concentration of membrane covered material in the cytoplasm is apparently still too great to be due solely to the nucleus, but it will not be possible to discuss the effect in detail until measurements are made at frequencies considerably higher than 16 million cycles. It has been interesting, however, to calculate by a series of approximations and extrapolations the order of magnitude of the "nuclear" membrane capacity to be $0.1 \mu\text{f}/\text{cm}^2$ at 8 million cycles.

In view of the interpretations given above, it might be quite instructive to observe the effect of high intensities of alternating current at

various frequencies It is easy to show that when the egg interior has the heat conductivity of water, it would be very difficult to maintain appreciable temperature gradients in the egg interior On the other hand, it seems that below 10 kc very little current penetrates the egg and any effects are due to heat from the medium At 100 kc there is a high potential difference and a large current flow across the plasma membrane At 1,000 kc the "nuclear" membrane is the controlling factor, while it will probably be necessary to go higher than 100 million cycles to be certain of a good nuclear penetration

It is not possible to estimate whether similar factors enter into the differences observed between diathermy and short-wave therapy without complete measurements on some of the tissues involved (*cf* Schereschewsky, 1926, Schereschewsky and Andervolt, 1928) Hemingway (1932) has shown that to within 2 per cent the body acts like a pure resistance but it should be pointed out that for *Asterias* suspensions the reactance at 8 million cycles is less than 10 per cent of the resistance and that it contributes less than 1 per cent of the impedance

We are very much indebted to Dr Hugo Fricke and Dr Howard J Curtis of the Walter B James Laboratory for Biophysics for the courtesy and cooperation which made this work possible

SUMMARY

The alternating current resistance and capacity of suspensions of unfertilized eggs of *Asterias forbesi* have been measured at frequencies from one thousand to sixteen million cycles per second

The plasma membrane of the egg has a static capacity of $1.10 \mu\text{f}/\text{cm}^2$ which is practically independent of frequency The suspensions show a capacity dependent on frequency at low frequencies which may be attributable to surface conductance

The specific resistance of the cytoplasm is between 136 and 225 ohm cm (4 to 7 times sea water), indicating a relatively high concentration of non-electrolytes

At frequencies above one million cycles there is definite evidence of another element of which the nucleus is presumably a part

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ELECTRIC IMPEDANCE OF ARBACIA EGGS

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In the first paper on this subject (Cole, 1928), the absolute value of the impedance of suspensions of *Arbacia* eggs was determined as a function of frequency of the measuring current and the following conclusions were given

"The specific resistance of the interior of the egg is about 90 ohm cm. or 3.6 times that of sea water and the impedance of the surface of the egg is probably similar to that of a polarization capacity. The characteristics of this surface impedance can best be determined by measurements of capacity and resistance of suspensions of eggs. No specific change has been found in the interior resistance or the surface impedance which can be related either to membrane formation or to cell division."

The finding of a static capacity, ϵ , a capacity which does not vary with frequency, for the membrane of the unfertilized *Hipponoe* egg, two and a half times this capacity for the fertilized egg, and a much higher equivalent internal resistance (Cole, 1935) made it desirable to measure both the resistance and the capacity of the *Arbacia* egg over as wide a frequency range as possible. The results on the unfertilized *Asterias* egg (Cole and Cole, 1936) further emphasized the necessity for such measurements.

Material

Live *Arbacia punctulata* were shipped from Woods Hole packed in seaweed and on ice. The ovaries were removed and placed in sea water or the injured animal was allowed to shed into sea water. The eggs were washed, centrifugalized lightly, and drawn into the conductivity cell, as was done for the *Hipponoe* and *Asterias* eggs. When a fertilized run was to be made, a few of the lower frequency points were measured on the unfertilized eggs. The suspension was then withdrawn, diluted with sea water, inseminated, washed to remove excess sperm, centrifugal

ized lightly, and returned to the conductivity cell. Over an hour was required for a complete frequency run and it was anticipated that the first cell division might complicate the experiments. It was found, however, that the electrical characteristics remained constant and that the eggs did not undergo first cleavage while in the cell. After diluting such a suspension with sea water most of the eggs went into the two cell stage but many stopped there and those which did proceed further developed abnormally. The material was not in especially good condition, but there was very little cytolysis.

The conductivity equipment of the Biophysics Laboratory, again available to us through the courtesy of Dr. Hugo Fricke, the other apparatus, corrections, and calculations are the same as for the *Asterias* work and are described in the preceding paper.

Data

The data for a typical unfertilized suspension are given in Table I and the complex plane locus plotted in Fig. 1. This locus is much the same as that of *Hipponoe* (as far as that went) and that of *Asterias*, except that the frequencies for corresponding points are higher. This is due in part to the smaller size of the egg and in part to the smaller capacity. The membrane capacity is again independent of frequency. The low frequency phenomena found for the *Asterias* egg are again present and the low frequency capacity is extrapolated in the same manner as before. In the data given, $C_0 = 140 \mu\text{f}$, so the membrane capacity $C_M = 0.74 \mu\text{f}/\text{cm}^2$ by the usual formula. The average value of C_M is $0.72 \mu\text{f}/\text{cm}^2$. A divergence similar to that for *Asterias* is sometimes found at the high frequency end of the circle. The calculation of the equivalent low frequency internal specific resistance leads to a value of 184 ohm cm . The average value is 186 ohm cm , or about 6 times sea water. The behavior of the curve at the highest frequencies is similar to that of *Asterias* except that it does not go so far, which makes an extrapolation to infinite frequency even more dangerous. Without attempting to assign any especial importance to it, this extrapolation leads to an average equivalent high frequency internal specific resistance of 120 ohm cm .

The data for a fertilized *Arbacia* suspension are given in Table II and Fig. 2 is the complex plane locus. It is much the same as for the fertilized *Hipponoe* eggs, but with the added high frequency tail. The extrapolated value of the low frequency capacity $C_0 = 560 \mu\text{f}$, so the static membrane capacity $C_M = 3.1 \mu\text{f}/\text{cm}^2$. These eggs gave

TABLE I

Suspension of Unfertilized Arbacia Eggs of Diameter 72 μ

Resistance of sea water, $R_W = 440.0$ ohms Volume concentration, $\rho = 53.7$ per cent Extrapolated capacity, $C_0 = 140 \mu\text{f}$ Cell constant, $K = 14.53$ Temperature, 21.7°C

Frequency		R_S	X_S
No	n		
		ohms	ohms
1	$1 \cdot 10^3$	1209.2	1.562
2	2	1209.3	2.843
3	4	1209.4	5.375
4	8	1209.3	10.64
5	$1.6 \cdot 10^4$	1208.4	20.60
6	3.2	1202.6	40.55
7	6.4	1190.0	75.45
8	$1.28 \cdot 10^4$	1146.0	125.0
9	2.56	1052.0	150.1
10	5.12	967.5	125.9
11	$1.024 \cdot 10^4$	916.0	79.15
12	2.048	893.0	49.15
13	4.1	882.8	39.90
14	8.2	871.9	48.15
15	$1.64 \cdot 10^7$	857.0	50.30

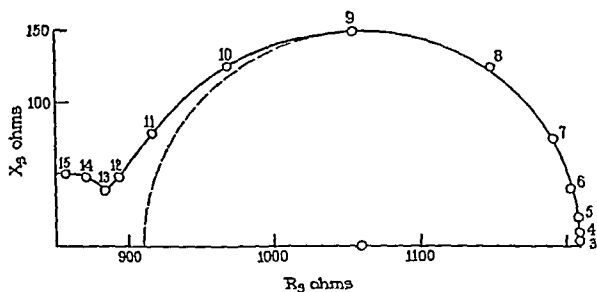


FIG. 1. Complex plane locus: series resistance, R_S , vs series reactance, X_S , for a suspension of unfertilized *Arbacia* eggs. Frequencies given in Table I.

TABLE II

Suspension of Fertilized Arbacia Eggs of Diameter 72 μ

Resistance of sea water, $R_W = 436.2$ ohms Volume concentration, $\rho = 42.8$ per cent
 Extrapolated capacity, $C_0 = 560 \mu\text{f}$ Cell constant $K_2 = 14.53$
 Temperature, 21.5°C

Frequency		R_S	X_S
No	n		
		ohms	ohms
1	$1 \cdot 10^3$	1025.0	4.01
2	2	1024.3	7.63
3	4	1023.4	14.83
4	8	1020.0	28.90
5	$1.6 \cdot 10^4$	1005.0	54.50
6	3.2	977.5	84.30
7	6.4	919.6	98.60
8	$1.28 \cdot 10^5$	866.0	83.60
9	2.56	826.0	57.22
10	5.12	808.0	47.45
11	$1.024 \cdot 10^6$	790.0	34.35
12	2.048	781.0	22.20
13	4.1	772.1	35.40
14	8.2	762.9	35.45
15	$1.64 \cdot 10^7$	752.4	32.95

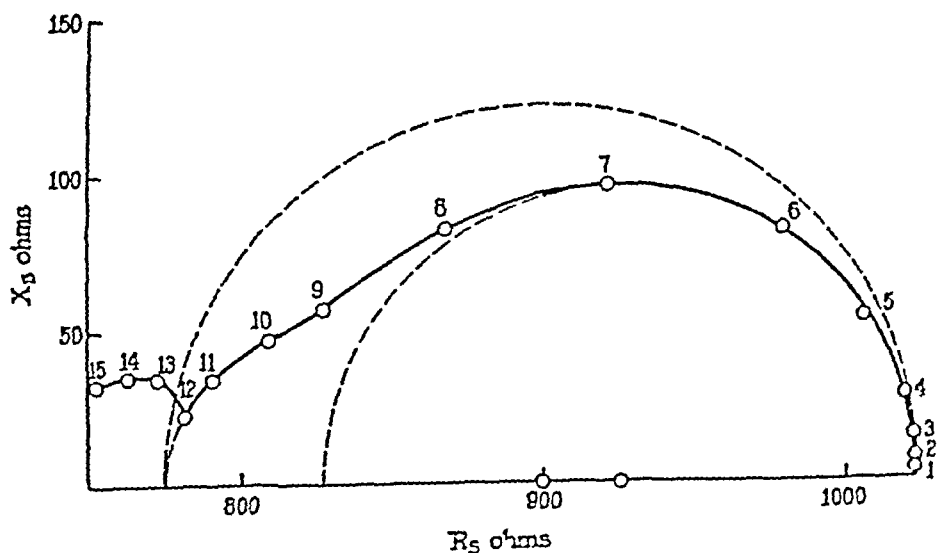


FIG. 2. Complex plane locus, series resistance, R_S , vs series reactance, X_S , for a suspension of fertilized *Arbacia* eggs. Frequencies given in Table II.

a value of $C_M = 0.73 \mu\text{f}/\text{cm}$ before fertilization, so the low frequency capacity increased about 4 times on fertilization. There was no change in the surface capacity effect at the lowest frequencies which could be correlated with fertilization. The low frequency data lie quite well on a circle up to the maximum reactance as was found for the fertilized *Hipponoe* eggs. The extrapolation of the high frequency end of this circle leads to an equivalent low frequency internal specific resistance of about 11 times that of sea water. The high frequency data are approximately the same as for the unfertilized egg.

DISCUSSION

Almost everything that was said of the unfertilized *Hipponoe* and *Asterias* eggs is applicable to the unfertilized *Arbacia* eggs. The static membrane capacity is about $0.72 \mu\text{f}/\text{cm}$ and the low frequency surface capacity effect is not qualitatively different from that in *Asterias*. The low frequency internal specific resistance is about the same—6 times that of sea water, and on the assumption of a highly conducting nucleus, its volume would be about 2 per cent of the egg volume. If the internal nuclear resistivity were the same as that of the cytoplasm, the nucleus would have a volume about 7 per cent that of the egg volume, and the resistivity of the cytoplasm would be 120 ohm cm or about 4 times that of sea water. The order of magnitude of such a "nuclear" membrane capacity is probably again $0.1 \mu\text{f}/\text{cm}$. The data of Harvey, 1932, give the nuclear volume as 0.4 per cent, so that on the basis of the above extrapolations the nucleus can only be a fraction of the material contributing to the high frequency effect.

The increase of static membrane capacity on fertilization seems to be somewhat larger for *Arbacia* than for *Hipponoe*, and the extrapolated value of the equivalent low frequency internal specific resistance, although high, is somewhat less for *Arbacia* than for *Hipponoe*. There seems to be no reasonable explanation of this increase of internal resistance on fertilization and no attractive interpretation of the course of the curve after it leaves the circle, so it will now be assumed that the internal resistance remains unchanged on fertilization. If then the membrane were nonconducting and had a static capacity independent of frequency, we would obtain the large dotted semicircle of Fig. 2. This may be taken as an indication that the internal conduct-

ance is unchanged and the departures from the unfertilized circle are due solely to the membrane. It is then possible to calculate the membrane capacity as a function of frequency, the result of which is shown in Fig. 3. The marked similarity of the form of this curve to a dielectric dispersion due to a dipole relaxation time is shown by the comparison in the figure with the theoretical curve calculated from the Debye theory (Debye, 1929). However, it has not been possible to take into account the energy dissipation which accompanies such a phenomenon, so this hypothesis cannot be taken too seriously until a mechanism can be proposed which will give a change of dielectric

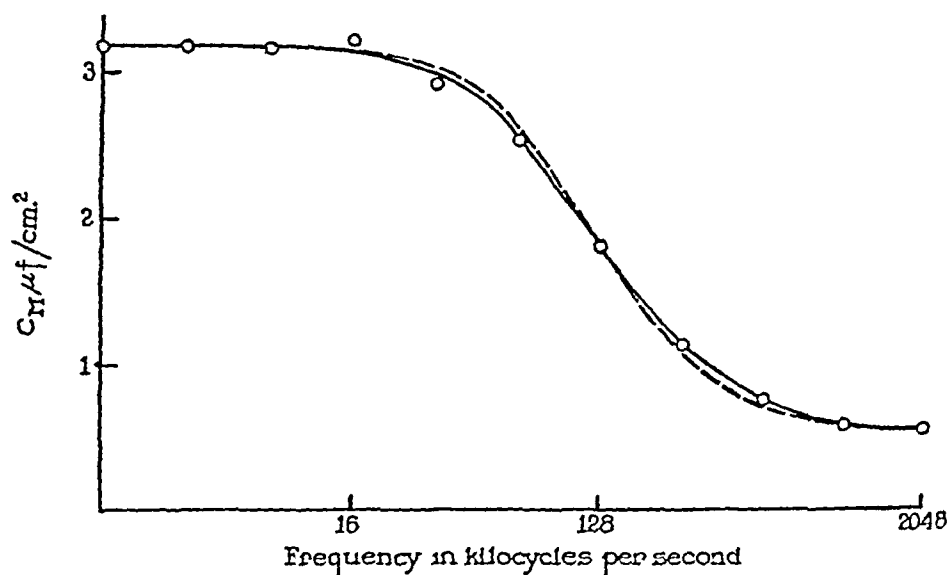


FIG. 3 Log frequency, n , vs membrane capacity, C_M , for fertilized *Arbacia* eggs

constant without dissipation. Another possibility will therefore be considered.

The outer portion of the fertilization membrane may be postulated to have a high static capacity, and be laid down at an appreciable distance from the plasma membrane of the unfertilized egg, while the interspace has the conductivity of sea water. Then at very low frequencies the current which does pass through the fertilization membrane will follow the layer of sea water rather than go through the plasma membrane and the capacity will be that due to the fertilization membrane alone. At higher frequencies, most of the current will

cross the plasma membrane and the cytoplasm, so the capacity will be that of the two membranes in series. Although a detailed analysis is not yet available, approximate calculations make this picture a very attractive one. The separation between the membranes would have to be 1.5μ for the *Arbacia* egg and somewhat less for the *Hipponoe* egg on this basis. The capacity of the plasma membrane is then $0.73 \mu\text{f}/\text{cm}^2$ and that of the fertilization membrane is $3.12 \mu\text{f}/\text{cm}^2$, so that high frequency capacity of the membranes in series should be $0.59 \mu\text{f}/\text{cm}^2$ whereas $0.55 \mu\text{f}/\text{cm}^2$ is the observed value.

The incomplete form of the data at the highest frequencies does not warrant any conclusions other than that there seems to be no qualitative difference between the unfertilized and the fertilized egg in this respect, and the speculations as to the cause of this effect are thus equally applicable.

In view of the wide disagreement between these conclusions and those drawn in the earlier paper, it is interesting to inquire as to the cause. Assuming an homogeneous egg interior, our present infinite frequency extrapolations give it an average specific resistance of 4 times sea water which is comparable with the value of 3.6 previously obtained. It is of course obvious that in the former work the frequency intervals were too great and the accuracy of the impedance measurement (1 per cent) not high enough to recognize the existence of the high frequency element. Even this is not sufficient to account for the rather highly dissipative polarization impedance previously found. The discrepancy has been investigated in some detail and on the basis of the present data it is not possible to explain the previous data in any way except to blame the material. This seems very unreasonable, since there is little doubt but that the material in the earlier work was in better condition than in the present case. It has occasionally been found that eggs which were in very poor condition gave a polarization impedance, but the *Hipponoe* eggs, which were in excellent condition, gave results consistent with the present ones.

On the other hand, it is comparatively easy to understand how the effect of fertilization was missed. In the first place, the series reactance is so small that there is never more than a 2 per cent difference between the series resistance and the absolute value of the impedance. Second, the frequency range where the effect of a change of capacity would be most marked occurs where the impedance is changing most

rapidly. A large capacity increase would have been observed except for the fact that in this particular frequency range the effective membrane capacity has fallen to about the same value as that of the unfertilized membrane.

We are very much indebted to Dr Hugo Fricke and Dr Howard J. Curtis of the Walter B. James Laboratory for Biophysics for the courtesy and cooperation which made this work possible.

SUMMARY

The alternating current resistance and capacity of suspensions of unfertilized and fertilized eggs of *Arbacia punctulata* have been measured at frequencies from 10^3 to 1.64×10^7 cycles per second.

The unfertilized egg has a static plasma membrane capacity of $0.73 \mu\text{f}/\text{cm}^2$ which is practically independent of frequency. The fertilized egg has a static membrane capacity of $3.1 \mu\text{f}/\text{cm}^2$ at low frequencies which decreases to a value of $0.55 \mu\text{f}/\text{cm}^2$ at high frequencies. The decrease follows closely the relaxation dispersion of the dielectric constant if the dissipation of such a system is ignored.

It is considered more probable that the effect is due to a fertilization membrane of $3.1 \mu\text{f}/\text{cm}^2$ capacity lifted 1.5μ from the plasma membrane, the interspace having the conductivity of sea water.

The suspensions show a frequency-dependent capacity at low frequencies which may be attributable to surface conductance.

The equivalent low frequency internal specific resistance of both the unfertilized and fertilized egg is about 186 ohm cm or about 6 times that of sea water, while the high frequency data extrapolate to a value of about 4 times sea water.

There is evidence at the highest frequencies that the current is penetrating the nucleus and other materials in the cytoplasm. If this effect were entirely due to the nucleus it would lead to a very approximate value of $0.1 \mu\text{f}/\text{cm}^2$ for the capacity of the nuclear membrane. The measurements do not indicate any change in this effect on fertilization.

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THE EFFECTS OF CURRENT FLOW ON BIOELECTRIC POTENTIAL

I VALONIA

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A description will be given of the effects of the flow of direct current, of controlled direction and density, upon the potential difference displayed across the protoplasm of impaled *Valonia* cells. The results are presented as string galvanometer records of the bioelectric potential before, during, and after current flow. Incidentally it is hoped that these will make more graphically evident some of the phenomena previously described in terms of resistance measurements with this organism¹.

It was concluded early in the study of these phenomena that the apparent resistance of the living protoplasm was largely due to the appearance of a counter E.M.F. ("polarization") opposing the applied potential, and that the observed variations with applied potentials were due to the magnitude and speed of development of this counter E.M.F. depending upon the direction, density, and duration of current flow. It is obvious that such a counter E.M.F. constitutes a change (increase, decrease, or reversal) of the P.D. previously existing across the protoplasm. While this change can be calculated from the change of apparent resistance and was so derived in a previous paper the P.D. is better measured and recorded in its own terms, e.g. as millivolts. The present records are designed to show this.

Furthermore, the counter E.M.F. often develops in a complex fashion, following a sigmoid or cusped time course during the first few seconds of current flow, in a manner difficult or impossible to follow by manual resistance settings. Finally, the form of the depolarization curve, occurring after the current has ceased to

¹ Blinks, L. R. *J. Gen. Physiol.*, 1929-30, 13, 793

² Blinks, L. R., *J. Gen. Physiol.*, 1930-31, 14, 139

flow, is often as interesting and characteristic as the polarization itself, this, of course, entirely escapes measurement in terms of apparent resistance

A few string galvanometer records of the course of changing potential during and after current flow were included in previous papers,^{1 2} these were, however, oriented to show increased "resistance" by a galvanometer deflection in a given direction regardless of whether the current passed inward or outward across the protoplasm. It was therefore not immediately apparent whether the normal $P D$ was being increased or decreased by current flow. The present records show this directly. In addition they present some new findings not previously described

Method

The principles of recording bioelectric potentials during current flow possibly call for brief discussion since such potentials are usually measured statically, with avoidance of current flow (at least in the measuring instrument). They merely involve balancing the purely ohmic resistance of the system (here largely that of the capillary inserted into the cell) so that the IR drop produced along this during current flow is compensated by an equal IR drop in the adjacent arm of the bridge, leaving only the changes of bioelectric potential to be recorded. This is conveniently done in the direct current bridge previously described,³ with vacuum tube detector. The latter may be regarded as an electrometer connected in series with the cell and one arm of the bridge, and shunted by the other two arms. In a bridge with equal ratio arms as here used, the shunt is equal to the series resistance, which reduces the electrometer sensitivity to half its open circuit value. Direct calibration of sensitivity is obtained under any given conditions by introducing a known EMF in series with the cell. This is frequently included in the records published, and from it is derived the effective millivolt sensitivity, marked as ordinates at the beginning of each record strip.

The records are photographs of the deflections of an Einthoven string galvanometer balanced into the plate circuit of the vacuum tube (201A worked at free-grid potential). The deflections of this instrument are essentially proportional over the field employed, and are practically rectangular at the film speeds used. This is shown by the calibrations taken with non-reactive circuits (e.g. dead cells, or those in the state of delayed polarization). With actively polarizing cells the deflections may be curved due to the change of current produced through the cell by calibration.

Since the bridge is necessarily a completed circuit, the cell's own potential can, of course, discharge continuously through it, in a "residual current." Although this might produce disturbing effects (as in *Nitella*, where special precautions are required), the $P D$ of *Valonia* is so small (usually less than 10 mv) that its discharge through the high resistances of the bridge (usually 50,000 to 100,000 ohms in series with the cell) produces only a small current, usually 0.1 or 0.2

³ Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 361

microampere, or in a cell of the average surface of 2 cm^2 a current density of $0.1 \text{ } \mu\text{a}/\text{cm}^2$, or less. This was in some cases abolished entirely by compensating the cellular E.M.F. to zero by an opposed series E.M.F. . Since the results taken in this manner differed in no appreciable way from those taken uncompensated, the residual current was allowed to flow during the experiments here reported. Its value is given in the legend of each figure. Such records have the advantage of showing the existing bioelectric potential in its positive or negative value at all times, before, during, and after current flow.

The current densities are derived from the estimated surface of the nearly spherical cells employed, an attempt being made to use cells of uniform size throughout, with a surface of about 2 cm^2 . There was necessarily, however, some variation in size and shape, so that the estimated surfaces are probably not correct to better than 10 or 20 per cent, with consequent variability of the current densities. Absolute values of these, however except to orders of magnitude, are not of very great importance since the threshold densities for various effects differ from cell to cell and from time to time. Much more significant are the relative values for the same cell; these are accurate to the agreement of the individual dry-cells of the bridge input battery, which were checked from time to time and replaced when their voltages became unequal. The relative densities are therefore accurate to 1 or 2 per cent.

Current densities are marked in $\mu\text{a}/\text{cm}^2$, the direction being indicated as "in" for positive currents passing inward across the protoplasm from sea water to sap, and "out" for those in the opposite direction. Sometimes upward arrows are used to designate inward currents, downward arrows for outward currents (on the records the counter E.M.F. develops counter to these arrows).

The current densities are to be regarded with two reservations.

(a) They are really increments or decrements produced upon the residual currents as a base, the latter are so small in *Valonia*, however, that they are negligible in comparison with most of the experimental densities employed.

(b) They are the densities at the beginning of current flow, therefore subject to decrease as the counter E.M.F. develops. The circuit conditions, however, render the extent of this decrease rather small, since to produce the necessary currents through the high resistances of the bridge (100 000 ohms or more), external potentials of 1 to 10 volts are often applied. The counter E.M.F. seldom exceeds 200 mv, so that the maximum reduction of current due to it is at most 20 per cent and usually much less; i.e., 2 or 3 per cent.

Positive potentials are shown below the zero line, negative ones above, in accordance with the convention followed in most of the bioelectric work from this laboratory. The sign is that of the outer surface of the protoplasm, a positive potential tending to produce positive current outward across the protoplasm.

The cells were impaled and supported in the arrangements previously described.^{2,4} KCl agar salt bridges led to calomel electrodes, non-polarizable at

⁴ Blinks, L. R., *J. Gen. Physiol.*, 1933-34, 17, 109.

the currents used. The basal ohmic resistances, used to balance the bridge, were determined from the values before impalement, and changed but slightly during the runs. They were further verified by A.C. measurements at 5,000 to 10,000 cycles (where the protoplasmic impedance was negligible) and in some cases, where polarization was completely delayed, by the resistance values of the impaled cell for low inward or all outward currents.

The results as given are believed to be typical of the genus *Valonia*. Both *V. ventricosa* and *V. macrophysa* were studied, the former in Florida, the latter in Bermuda, with essential agreement. The Bermuda species appears to be somewhat more hardy, living longer in the laboratory, particularly when impaled (e.g. Fig. 9).

Measurements were usually made on cells which had stood at least a day or two after impalement in order to insure manifestation of the full bioelectric potential and effective resistance, by healing of the wound inflicted by insertion of the glass capillary. The necessity for this precaution is obvious and has been repeatedly emphasized. The results obtained soon after impalement will depend upon at least two factors: the previous state of the cell (i.e. whether displaying delayed or regular polarization as defined below), and the extent of the leaks around the capillary, which will short circuit the transprotoplasmic circuit and reduce the apparent magnitude of both the P.D. and the polarizations to an extent depending upon the relative areas of injured and uninjured protoplasm. As the wounds heal (for migration of chloroplasts, and other morphological details in this process, see the recent reports of Kopac⁵) an intact surface is sooner or later re-established. There are, however, secondary changes usually supervening, apparently involving the whole protoplasmic surface, and producing if it was not already present, the state known as variable resistance or delayed polarization. This will be taken up below. Whether this state (or any state of the impaled cells for that matter), is "normal," seems increasingly futile to discuss. It might be concluded that the "constant" state, with its prompt and regular polarizations and high effective resistance, is a normal one, reflecting, for example, the relative impermeability of the cell to electrolytes. This is borne out by its almost invariable occurrence in other plant cells so far studied (5 other genera). But it has now proved possible to produce or destroy this state at will in *Valonia*, by proper chemical treatment, without having to wait for time, "injury, recovery" etc., to accomplish the changes. This is more likely to throw light upon the structural and metabolic factors involved than academic discussions of normality. It seems reasonable to assume instead that both states are functional, occurring spatially or temporally in the same cell according to the prevailing internal or external milieu, especially, as shown below, acidity. The ease with which one state passes into the other may have an important bearing upon other problems, e.g. the accumulation of electrolytes, since instead of a constant surface for penetration, there may be one

⁵ Kopac, M. J., *Carnegie Institution of Washington, Year Book* No. 32, 1932-33, 273, No. 33, 1933-34, 253.

which changes its properties depending upon which type of ion—acidic or basic, strikes it at a given place.

The essential point is, however, that the regular and delayed polarization states differ, not merely quantitatively (depending for instance on the relative areas of injured and uninjured surface⁵) but fundamentally and qualitatively in their time relations in a manner to be distinguished only by control of current density and by continuous records of the time course of polarization. These differences are best shown by the actual records which will now be presented.

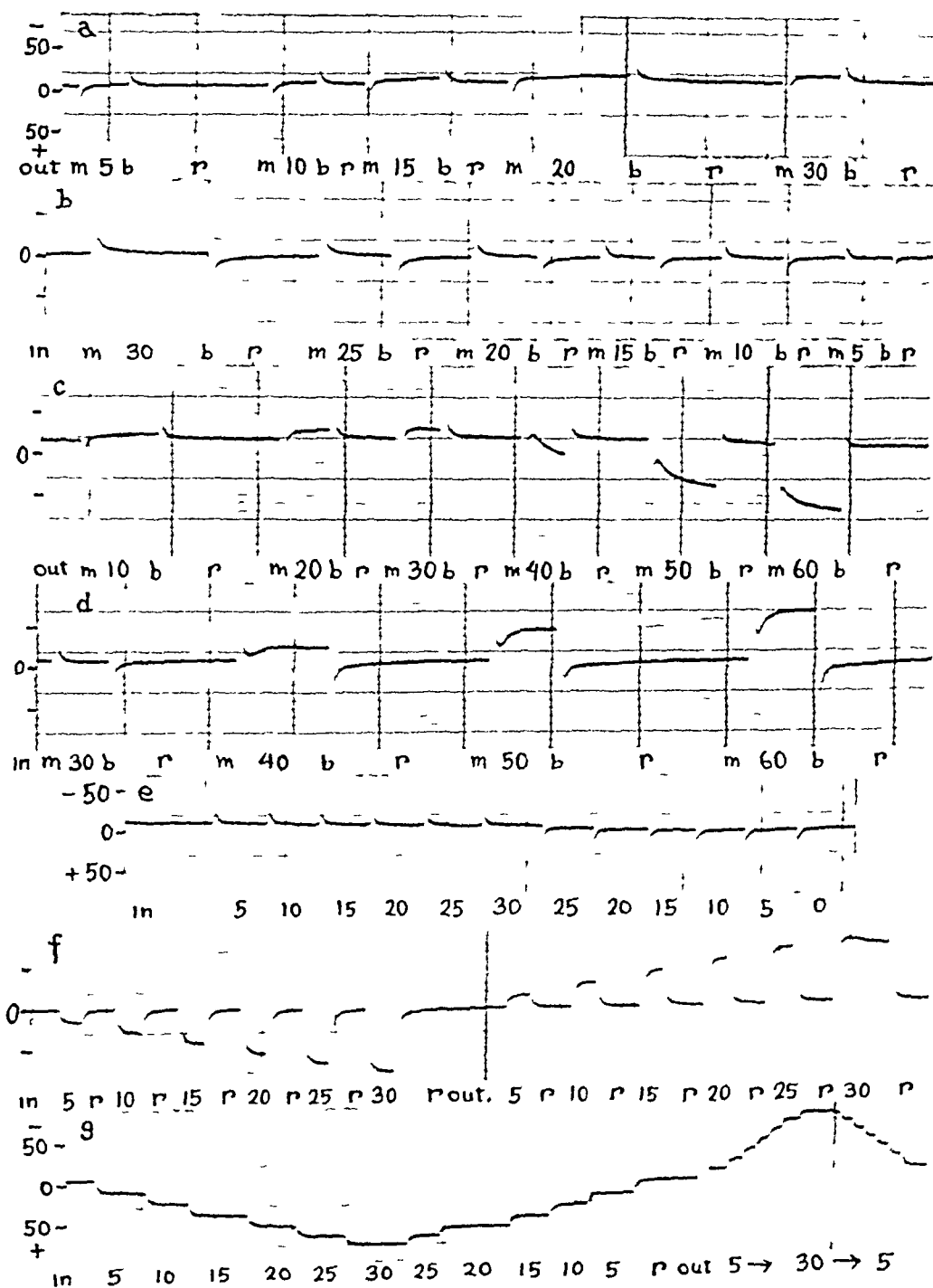
Types of Response to Current Flow

Several stages of electrical behavior were distinguished in *V. ventricosa*,¹ where an attempt was made to relate them to degrees of injury and recovery after collecting, impaling, etc. Most of the same characteristics are displayed by *V. macrophysa*, but further study has shown that they may be controlled to a large extent by experimental treatment, which will be described below.

Two principal states are distinguished (A) "regular," and (B) "delayed" polarization, the latter term being used for convenience without prejudice as to the real nature of the counter E M F (i.e. whether it has the character of a static or a polarization capacity, there is evidence, partly given below, that it is largely polarization). These may be defined as follows:

(A) *Regular Polarization*—There is an immediate and regular development of counter E M F, beginning at the instant of current flow, and being proportional, at least for the smaller currents, to the current density, in either direction across the protoplasm. This gives rise to an effective resistance which is high and uniform for these current densities, and corresponds to the state of "constant resistance" earlier described.³

(B) *Delayed and Non Proportional Polarization*—There is little or no counter E M F developed with small currents in either direction across the protoplasm, nor with very large ones passing outward, but a large counter E M F develops suddenly, with a sigmoid curve, when a critical or threshold density of inward current is reached. After this has occurred, larger currents produce only slightly greater counter E M F's, but the protoplasm has become thereby "conditioned" so that polarizations occur thereafter, not only with considerably smaller



inward currents, but even, temporarily, with outward currents. This corresponds to the state of "variable resistance" previously described.¹

These two states merge into each other, delayed polarization passing spontaneously over into regular polarization through intermediate stages where some of the characteristics of each are displayed. Each sometimes occurs, however, in a pure form which will first be described.

Regular Polarization

This condition is found in a few freshly gathered cells, and in most of those which have stood undisturbed in the laboratory for some days.

FIG. 1. Effects of current flow on the P.D. of a cell of *Valonia ventricosa* which had reached the constant resistance level and was then impaled, the records being taken within a few minutes after impalement. The P.D., about 5 mv negative (inwardly directed), is low, as is also the effective resistance (the latter being only 750 ohms higher than that of the capillary alone), this is due to the electrical leak around the insertion of the capillary, still unhealed.

Records *a* to *e* inclusive were taken with the bridge balanced to this effective resistance. The string image, after a deflection at 'make' (*m*), returns to zero in the steady state, it does the same again after a deflection at 'break' (*b*). The deflections are seen to be regular and symmetrical for both inward (*in*) and outward (*out*) currents up to some $30 \mu\text{a}/\text{cm}^2$ of cell surface. Above this (Records *c*, *d*) there is an increasing recession after a cusp, the steady state during current flow being away from the zero line, indicating bridge unbalance (cell resistance reduced). It should be noted that the recession is somewhat larger for outward currents (*c*) than for inward (*d*)—this is practically the only difference between them in this state.

Record *e* was taken with the bridge balanced as before to the effective resistance, but 6 equal increments of $5 \mu\text{a}$ each were made without a break, then 6 equal decrements down to zero again. The essential similarity of each response is evident.

Records *f* and *g* were then taken with the bridge balanced only to the ohmic resistance of the system (largely the capillary) here about 14,000 ohms. The counter E.M.F.'s now build up as positive or negative potentials respectively for inward and outward currents in *f* with a break (to *r*) between each value of current density followed by a return to zero (*m*' and *b*' are omitted here) in *g* without a break between the increments and decrements. Sensitivity about 10 mv per division, zero and 50 mv + and - being indicated on each record. Residual current *r* (due to discharge of bioelectric potential when no external E.M.F. is applied), about $0.15 \mu\text{a}/\text{cm}^2$. Time marks 1 second apart.

Current densities in μa per cm^2 of cell surface, the experimental densities being shown with figures, the residual current on cessation of flow, by *r*.

or weeks after cleaning and separating. When such a constant cell is impaled, it usually continues to display for a while its regular polarizability, although reduced in magnitude because of the short circuiting introduced by the injured zone around the capillary. An example is shown in Fig 1, taken immediately after the impalement of such a cell. When the bridge is balanced to the effective resistance of the system (here only 750 ohms higher than the capillary), the polarizations appear as "kicks" upward or downward at make and break of current, approaching the zero line in the steady state except with too high currents, when they show a cusp and a recession (*c* and *d*, Fig 1). When the bridge is balanced only to the ohmic resistance involved, then the polarizations appear in their true light as alterations of the existing (here very low) bioelectric potentials. Recessions from the highest value begin to appear slightly sooner for outward currents than for inward ones, this is practically the only difference between them. The regularity and symmetry of curves in charge and discharge, and in successive polarizations, should be noted.

In a few cases (particularly where a very fine capillary has been inserted) this regular polarization persists through the life of the cell while impaled, the polarizations merely becoming larger, and the effective resistance higher, as the injured area heals. Usually, however, the cells pass over into the variable or delayed state, which will next be described.

Delayed Polarization

Although this may not be a normal condition (*e g* it may reflect a heightened permeability to electrolytes) it is nevertheless almost universally found in freshly gathered cells, and in most impaled ones, which are able to survive for a long time in this condition. To be sure, the state is not always pure, since there is often a small polarization to low currents, becoming disproportionately larger at a certain threshold of inward current. An example of this is shown in Fig 2, taken with the same cell used for Fig 1, but 10 days after impalement. This may be taken as a representative of the transitional state, referred to again later.

There are, however, many cells which display a pure state of delayed polarization, showing no polarization at all with small currents. These

cells are like purely ohmic conductors, without reactance, and with a resistance practically that of dead cells to all outward currents, and to inward currents up to the threshold density. An example is shown in Fig. 3, where it is impossible to distinguish make and break of current except by the rectangular deflection due to a slight ohmic unbalance in the bridge. (This serves, by the way, to show the absence of spurious reactances in the system, such as electrode polarizations.) Other, even better examples of the lack of polarization at low current densities are seen in Fig. 13.

If the inward current be sufficiently increased, however, a critical density is reached at which a large polarization is suddenly produced. The threshold for this effect varies somewhat from cell to cell, and with time in the same cell. It often lies at about $25 \mu\text{a}/\text{cm}^2$ but may be much higher, especially with the first flow of current after a long rest. In Fig. 3, for example, it is between 40 and $45 \mu\text{a}/\text{cm}^2$, about as high as it ever goes. The time course of the response is distinctly sigmoid, with a slow start and an inflection to a very rapid rise of positive P.D. There may or may not be a cusp at the apex of this curve before it flattens out to a steady value, the cusp is lacking in Fig. 3, but is shown in other records (Figs 5d, 8, 9, 15). Obviously the original bioelectric potential has been reversed, and remains reversed as long as the current continues to flow. When the current is stopped this positive P.D. rapidly decreases, then more slowly returns to a negative value in a smooth curve.

This performance may be repeated any number of times, essentially the same positive potential being reached each time, but the curve changes shape, becoming progressively faster with equal current flows in rapid succession, so that the inflection may smooth out and practically disappear. Examples of this are shown in Fig. 4. On the other hand if a long wait intervenes between flows, the curve again becomes lower (Fig. 5). The duration of current flow also affects the speed of subsequent depolarization, negativity being regained more quickly after short flows than after long ones. This is shown in Fig. 4. The polarization curve is also usually different in shape from the depolarization.

In all these respects the response at the threshold differs from either condenser discharges or electrode polarizations, since in neither of these

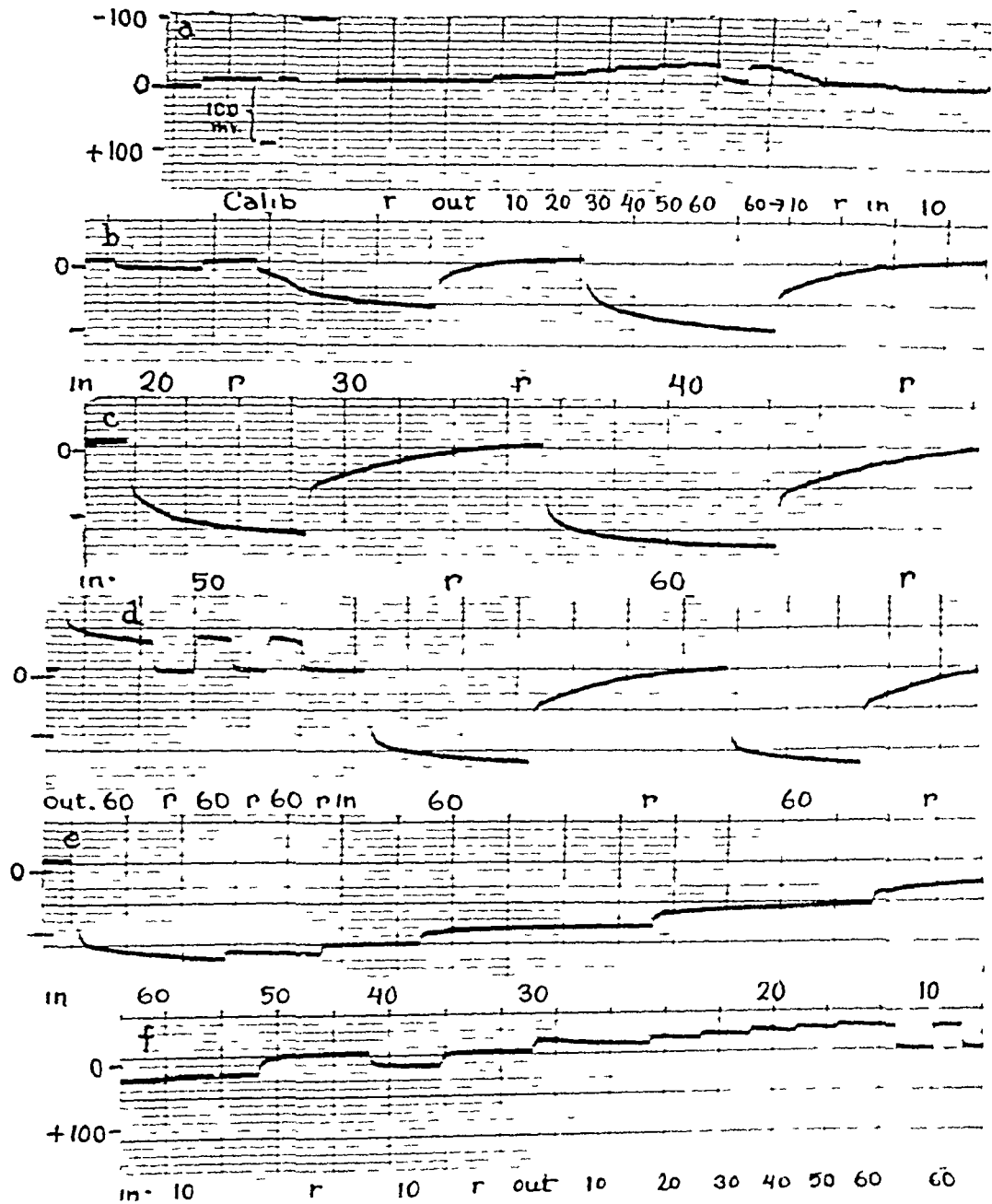


FIG 2

is there such an effect of one current flow upon the next. This is the first of the conditioning effects to be met with in the delayed state. Another will now be taken up.

If the current density be changed from the threshold value, several interesting results occur. An increase usually produces a still higher positive P.D., as seen in Fig. 3 for example. But the extent of this increase is not at all proportional to that produced at the threshold and two or three current increments usually cease to increase the positive P.D., at least beyond a temporary cusp, which is followed by a recession, sometimes even to lower values than at the threshold density itself (Fig. 7c).

On the other hand decreased inward current densities now produce a much larger effect than before the threshold flows, good polarizations

FIG. 2. Effect of current flow on the P.D. of *Valonia verticosa* the same cell as used in Fig. 1 but 10 days later. At the start the bioelectric potential is about 10 mv. negative (i.e. inwardly directed). (Marks as in f and g Fig. 1 but r is omitted in some cases.)

Outward currents in 6 equal increments (without breaks) up to $60 \mu\text{A}/\text{cm}^2$ increase this P.D. to about 35 mv. negative; this is followed by 6 equal decrements. Larger outward currents have little further effect. Inward currents first decrease the negative P.D. driving it to zero at $20 \mu\text{A}/\text{cm}^2$ and at a threshold of $30 \mu\text{A}/\text{cm}^2$ reversing it to large positive values, but with a delay giving a sigmoid time course. Recovery to negative values occurs on interruption of current (to residual current r). Further increments of inward current increase the P.D. up to about 150 mv. positive. An outward current of the same density produces a much larger counter E.M.F. than in Record a but this rapidly recedes to a lower value which is duplicated on succeeding outward flows. After two interrupted inward flows of $60 \mu\text{A}$ the current is decreased in 6 equal decrements without interruption giving the effects of Records e and f.

It should be noted that $20 \mu\text{A}$ maintains a much higher positive P.D. than it originally produced (b) and that even $10 \mu\text{A}$ sustains positive P.D. for some time although steadily falling off in value and becoming still less on a succeeding flow after short interruption.

The non proportionality of response to equal decrements should be noted in contrast to the regular state of Fig. 1 as well as the hysteretic or conditioning effect of inward current, which passes off after large flows, as shown by the small final response to outward currents.

Sensitivity about 12 mv. per division, zero and 100 mv. being indicated on each record (+ and - calibration on a). Residual current (r) about $0.15 \mu\text{A}$ per cm^2 of cell surface. Time marks 1 second apart.

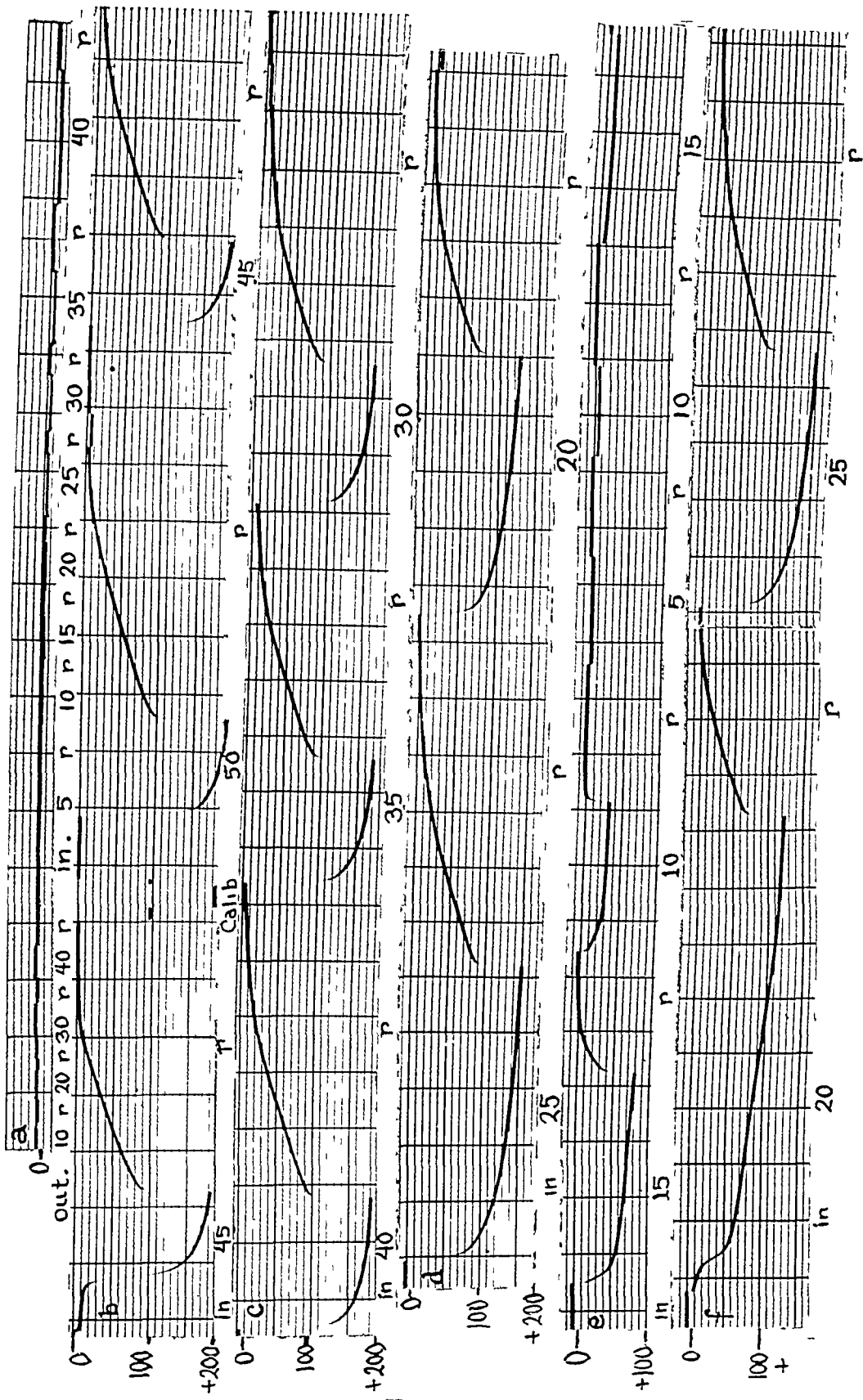


FIG 3

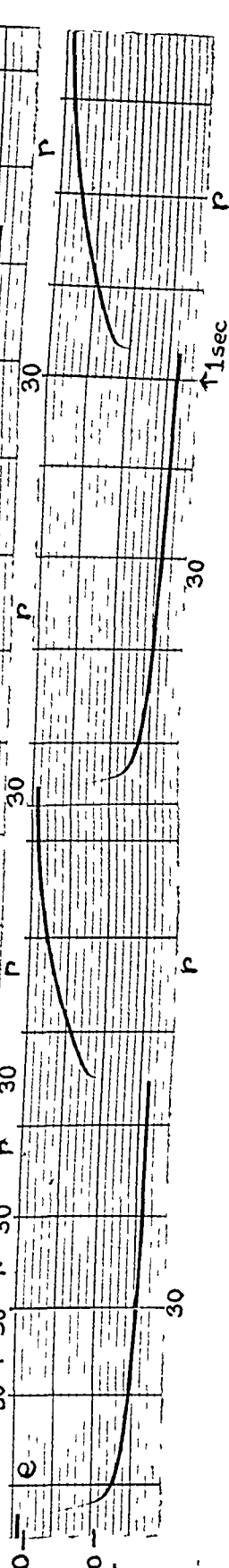
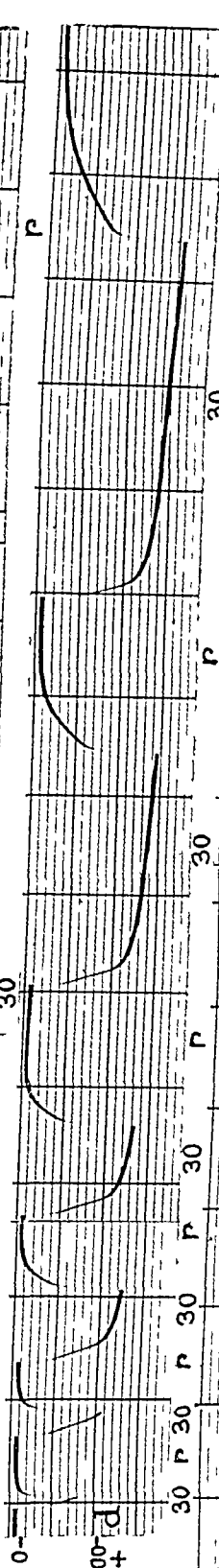
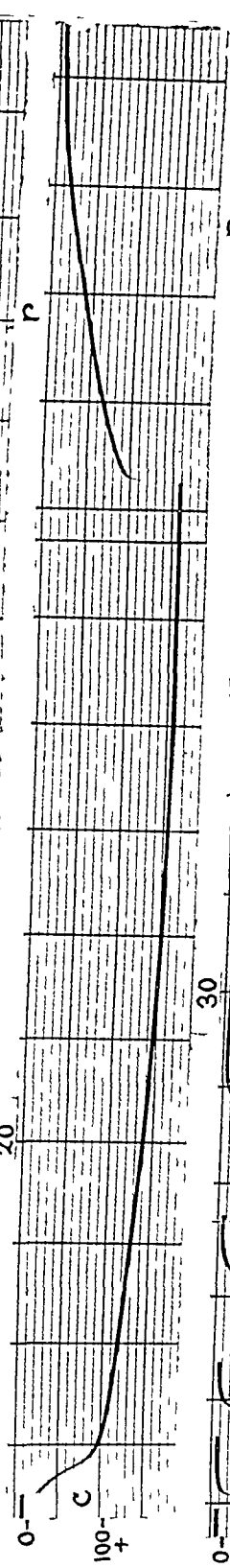
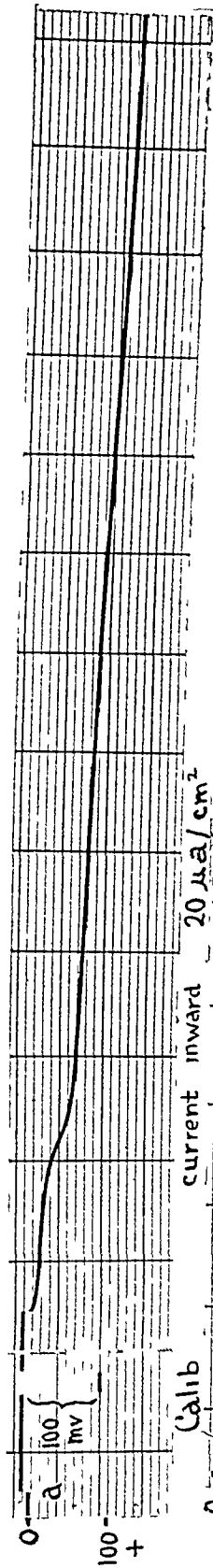
being elicited down to 10 or even $5 \mu\text{a}/\text{cm}$ in some cases. The lowest densities produce, however, proportionally less effect than the higher ones, and the response tends to die away, either on continued flow, or on successive passages of current. Some of these effects are shown in Fig. 3, a lowering of the threshold for later current flows is also to be seen in Figs 5, 8, and 13.

Another type of record bearing on the non proportionality of response in the delayed state is shown in Fig. 5, where step wise increments or decrements of continued inward flow are employed. The rather large changes at the threshold density compared with the much smaller ones above this are clearly shown, as well as some of the 'hysteric' effects by which polarizations are maintained, after larger flows, by densities originally unable to produce them. The gradual dying away of this condition during the continued flow of inward current ($10 \mu\text{a}$) is also shown, as well as a progressive fall of threshold from $60 \mu\text{a}$ down through 50 (Record *d*) to 30 (Record *f*) and even to $20 \mu\text{a}$ (Record *g*). $10 \mu\text{a}$, however, is never able to maintain positivity for long.

One might say that the passage of the threshold polarizing current has conditioned the protoplasm to a regularly polarizing state, for the curves shown in Fig. 5, and perhaps even better in Fig. 8 for $5 \mu\text{a}$ increments or decrements in the mid range of densities (*e.g.* 10 to $25 \mu\text{a}$ total flow) are extremely regular, reproducible, and symmetrical,

FIG. 3 Characteristics of fully delayed polarization in *V. macrophysa* (marks as in *f* Fig. 1). The cell (impaled about 3 weeks) is extremely non reactive to lower current densities displaying practically no counter E.M.F. to currents up to $40 \mu\text{a}/\text{cm}^2$ in either direction. Only at a threshold of $45 \mu\text{a}$ does counter E.M.F. develop and here very abruptly to 200 mv positive P.D. The conditioning effect of such flow is however shown by the succeeding records taken after an increase to $50 \mu\text{a}$ with progressively smaller inward currents. Good polarizations are elicited down to $15 \mu\text{a}$ but much weaker at 10 and practically none at $5 \mu\text{a}$. Increases again through 15 are not very effective the threshold being $20 \mu\text{a}$ where a sigmoid reversal curve results. The sigmoid character is again nearly lost at $25 \mu\text{a}$.

Sensitivity about 11 mv per division, with values indicated on each record and calibrations on *b*. Residual current (*r*) about $0.05 \mu\text{a}/\text{cm}^2$. Time marks 1 second apart.



both for charge and discharge (increments and decrements) and for successive current flows (equal changes). This distinctly resembles the state of affairs in regular polarization except that here the inward conditioning current must continue to flow, with small changes made in its value.

Indeed, the conditioning sometimes lasts long enough for fairly good polarizations to be produced to actual outward flows. This is a third effect of one current flow upon succeeding ones. In Figs 2, 6*b* and 7 are shown such temporary polarizations to outward currents following closely on inward ones. But in every case, there is only a cusp, with a quick recession and successively smaller polarizations to later flows. This may be due to the spontaneous loss of polarizability, which occurs even during the continued flow of small inward currents (e.g. Fig 5). But the process is speeded up, as evidenced on records not here shown, by the flow of outward current itself. The "deconditioning" effect of outward current is likewise shown by the subsequent behavior of inward currents, for which the threshold may again be somewhat raised, although usually only the time of response is increased (Figs 6 and 7), a more elongated sigmoid curve resulting.

Transitional States

Fig 2 showed a transitional state of a cell which had formerly been regularly polarizing, but later displayed some of the characteristics of delayed polarization such as a threshold where inward current gave rise to suddenly increased positive P.D. in a sigmoid curve. As cells

FIG 4 Records showing the increasingly rapid response obtained with successive inward currents in *Valonia macrophysa* impaled over 2 weeks (marks as in *f* Fig 1). The threshold in this cell is $20 \mu\text{A}/\text{cm}$ but the response is extremely slow at this density. A sigmoid curve is still obtained when the density is increased to $30 \mu\text{A}$ becoming less and less pronounced on succeeding flows in Record *d* and practically smoothed away in *e*. The speed of depolarization also depends upon the length of time the current had flowed being fastest after short flows slowest after long ones. (1 second intervened at the spot marked on Record *e* shortened to place it on the page.)

Sensitivity about 11 mv per division zero and 100 mv positive being marked on each record derived from the calibration on *a*. Time marks 1 second apart. Residual current (*r*) about $0.1 \mu\text{A}/\text{cm}$.

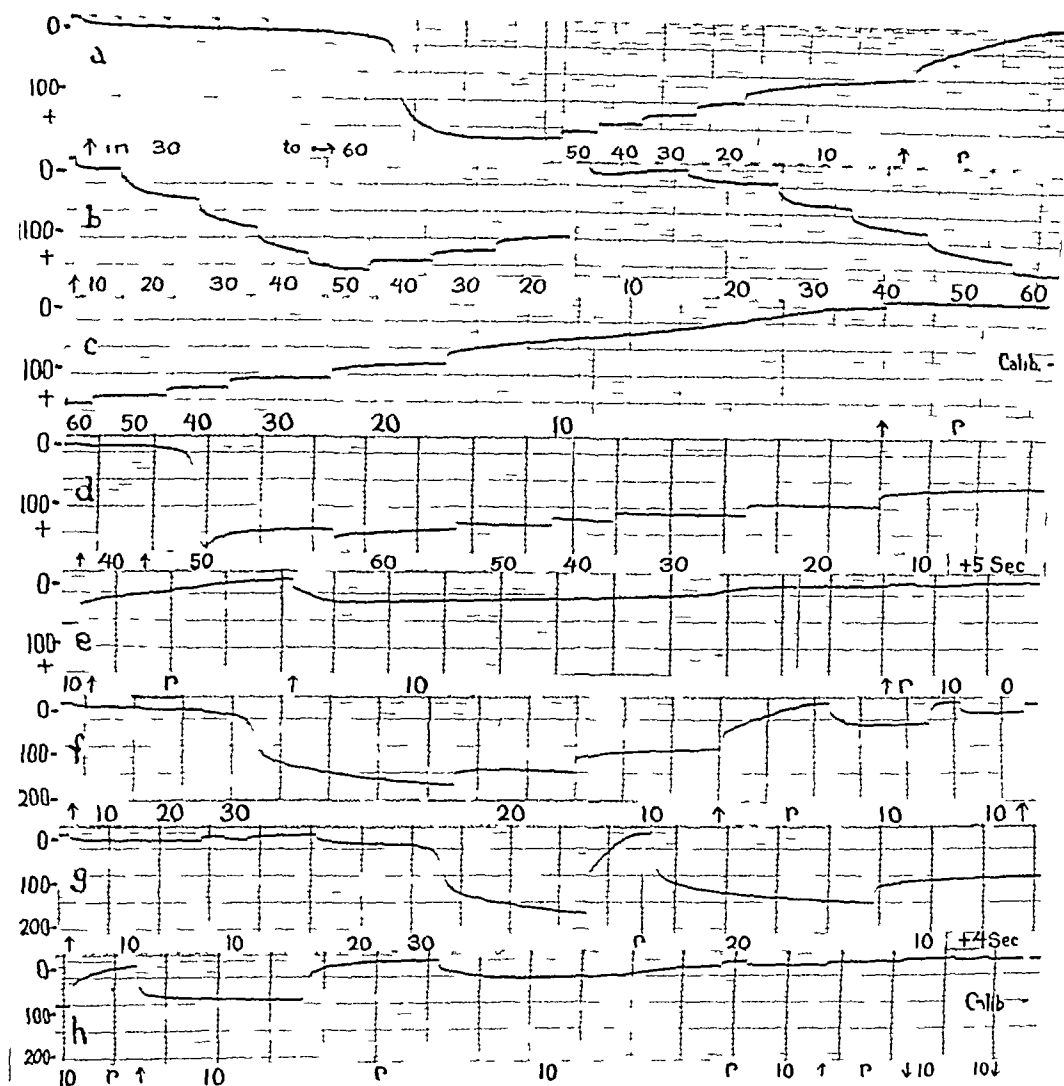


FIG 5 Further characteristics of delayed polarization in *V. macropphysa* (cell impaled 2 weeks). Polarization is first elicited on increasing the inward current from 30 up to 60 $\mu\text{A}/\text{cm}^2$. 10 μA decrements then produce nearly equal decrease of P_D down to about 20 μA , where recession begins, and becomes very marked at 10 μA , this density being unable to maintain positive potentials, as shown on several of the records. On the other hand 20 μA becomes increasingly capable of maintaining positivity as a result of the conditioning effect, the threshold first falling to 30, then to 20, and eventually (in *h*) to 10 μA , although a brief interruption here is followed by recession. Small polarizations to 10 μA outward flow conclude the record.

Sensitivity about 8 mv per division in Records *a* to *e* inclusive, reduced to 12 mv per division in *f* to *h*. Residual current (r) about 0.1 $\mu\text{A}/\text{cm}^2$. 100 mv calibrations on *c* and *h*. Time marks 1 second apart. Marks as in *f* and *g*, Fig 1. Upward arrows signify inward, downward arrows, outward currents.

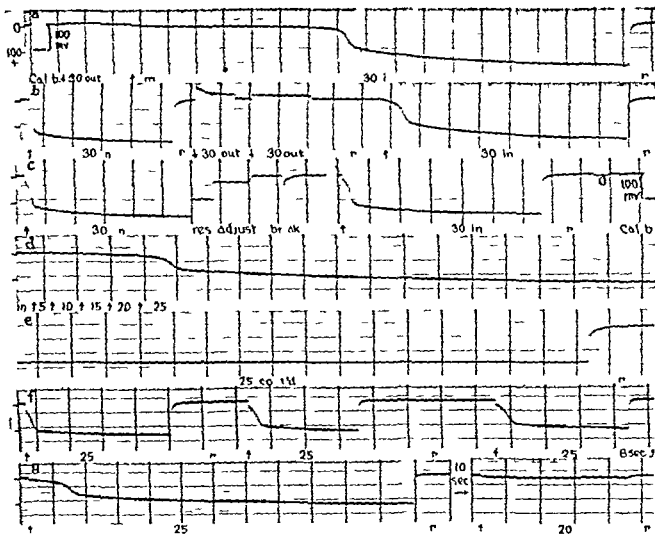


FIG 6 Further characteristics of delayed polarization in *V. macrophysa* (same cell as Fig 5) especially in relation to the time factors (Marks as in Fig 1) The threshold for inward current polarization is $30 \mu\text{A}/\text{cm}$ the sigmoid inflection occurring only after about 7 seconds continuous flow. A second passage of this current however produces rapid effects the inflection being almost smoothed away although still perceptible in *b*. Following this $30 \mu\text{A}$ outward current produces an appreciable counter EMF which rapidly recedes and on second exposure remains low. After this deconditioning by outward current the time course to inward current is somewhat lengthened (*b*) to become again shortened on subsequent flows in Record *c* which also shows the effects of adjusting the bridge balance from the true ohmic value of the capillary (here 20 700 ohms) to the effective resistance of the cell plus capillary totaling 23 100 ohms. Two steps of 1000 and 4 of 100 ohms each are shown, bringing the galvanometer to zero. The current is then interrupted the typical 'break' deflection being produced much as in Fig 1. It is seen to have exactly the same shape as the depolarization curves preceding and succeeding it with the same current density. (Ohmic balance was restored before the second half of *c*.)

Following on these $30 \mu\text{A}$ flows the current was built up (*d*) through 5 equal increments of $5 \mu\text{A}$ to $25 \mu\text{A}$ at which density a response was obtained although reaching a steady value extremely slowly (*e*). This current was then interrupted for varying lengths of time 2.5 and 4 seconds on *f* and 8 seconds between *f* and *g* the decreasing speed of response is noticeable. Finally after 11.3 seconds interruption, $20 \mu\text{A}$ is seen to evoke very slight response.

Sensitivity about 11 mV per division with zero and 100 mV positive being marked on each record. Calibrations on *a* and *c*. Time marks 1 second apart. Residual current (*r*) about $0.1 \mu\text{A}/\text{cm}$. Upward arrow signifies inward downward arrow outward current.

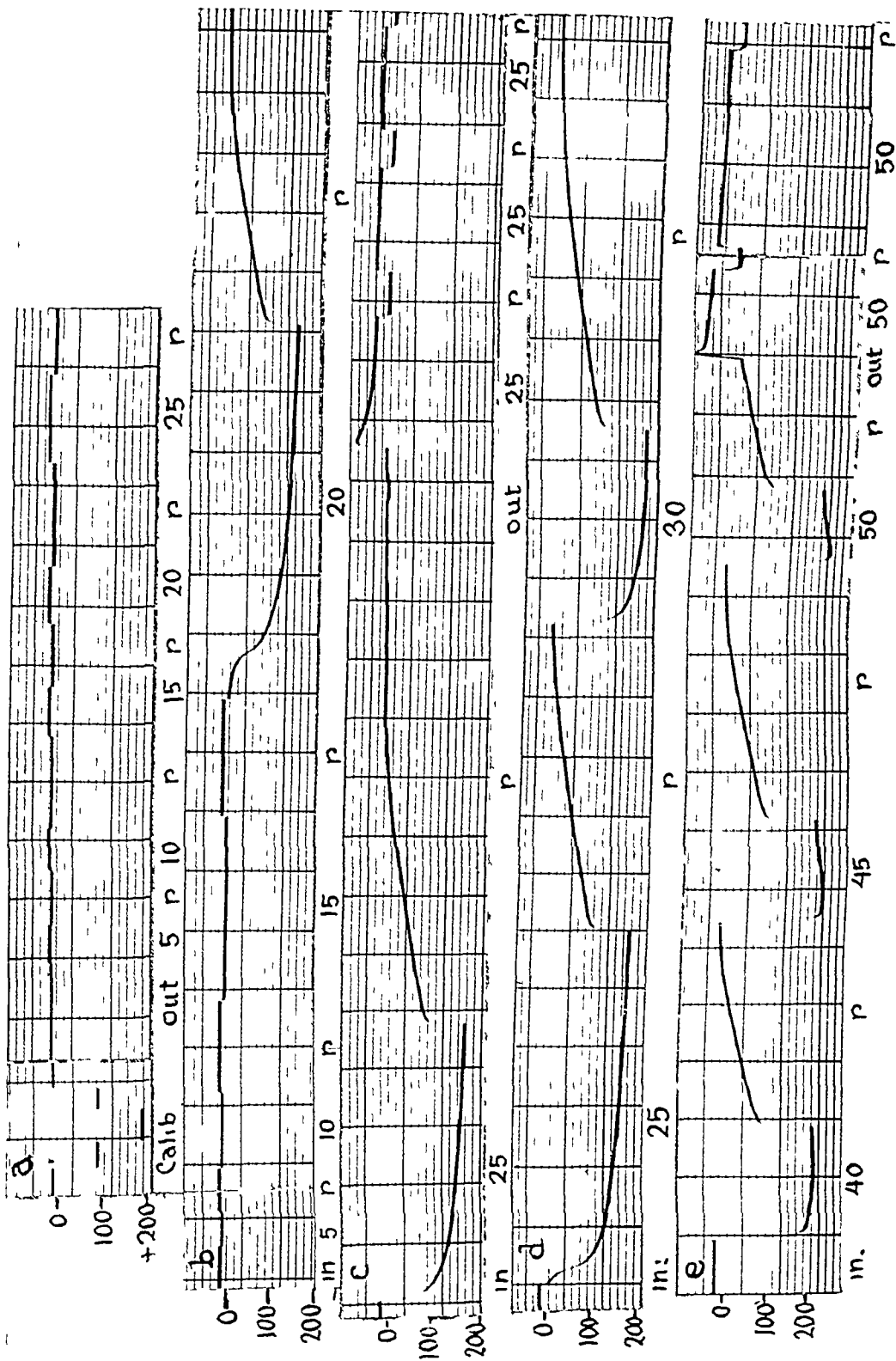


FIG. 7

stand quietly in sea water after impalement, they tend spontaneously to pass over through such transitional stages to regular polarization again. One of the invariable signs of this transition is the production of a more or less permanent positive $p.d.$ as the result of current flow. We have already seen many examples of a tendency to linger at positive values, the more pronounced after long current flows. Fig. 9 shows this tendency exaggerated to an eventual flattening out of the $p.d.$ to a temporary positive value, after increasing inflections. When this has occurred, positivity is often retained even after outward flows of high density, as shown in Fig. 9 *c* and *d*. Upon this positive $p.d.$ as a base, very characteristic polarizations are now produced, small currents in either direction producing immediate and very regular counter EMFs, which become more and more cusped with increasing density. Another characteristic is the tendency for polarizations with outward currents to become even larger than the corresponding ones to inward currents (Fig. 9 *f*) so that the resistance is greater to outward than to inward currents, quite contrary to the usual situation. Eventually, the positive $p.d.$ is seen to disappear, and with it these characteristic of polarization.

FIG. 7. Characteristics of the delayed polarization state in *Valonia microphysa*. There is only a slight counter EMF developed with outward currents up to $25 \mu\text{A}$ per cm^2 of cell surface, and very little more with inward currents up to $15 \mu\text{A}$. At $20 \mu\text{A}$ however, strong polarization develops with a sigmoid curve. At break there is an abrupt drop of positive $p.d.$, followed by a slower, nearly linear approach to zero and the original negative $p.d.$ The same is true for larger inward currents except that the speed is increased, the sigmoid curve becoming smoothed out. After $25 \mu\text{A}$ inward flow, the current was reversed, the temporary polarization to outward current, which is rapidly decreased and not recovered in succeeding flows is characteristic. So also is the same but slower time curve, with sigmoid course, produced by $25 \mu\text{A}$ inward flow following on these outward flows. It may be noted as a further characteristic that the higher inward current densities (40 – $50 \mu\text{A}$) produce little if any greater permanent effect, the first increase being succeeded by a reaction to a nearly constant level (here at about 200 mV positive $p.d.$). Finally there is a temporary polarization with $50 \mu\text{A}$ outward current, followed by a rapid recovery, and still less response on a second flow.

Sensitivity about 16 mV per div (on) with zero and 100 mV (off) and 200 mV values shown on each record derived from the calibrations on a Time marks 1 second apart. Residual current (r) $0.05 \mu\text{A}/\text{cm}^2$ inward, or 1 per cent of the lowest experimental current density here used. Marks as in *f* and *g*, Fig. 4.

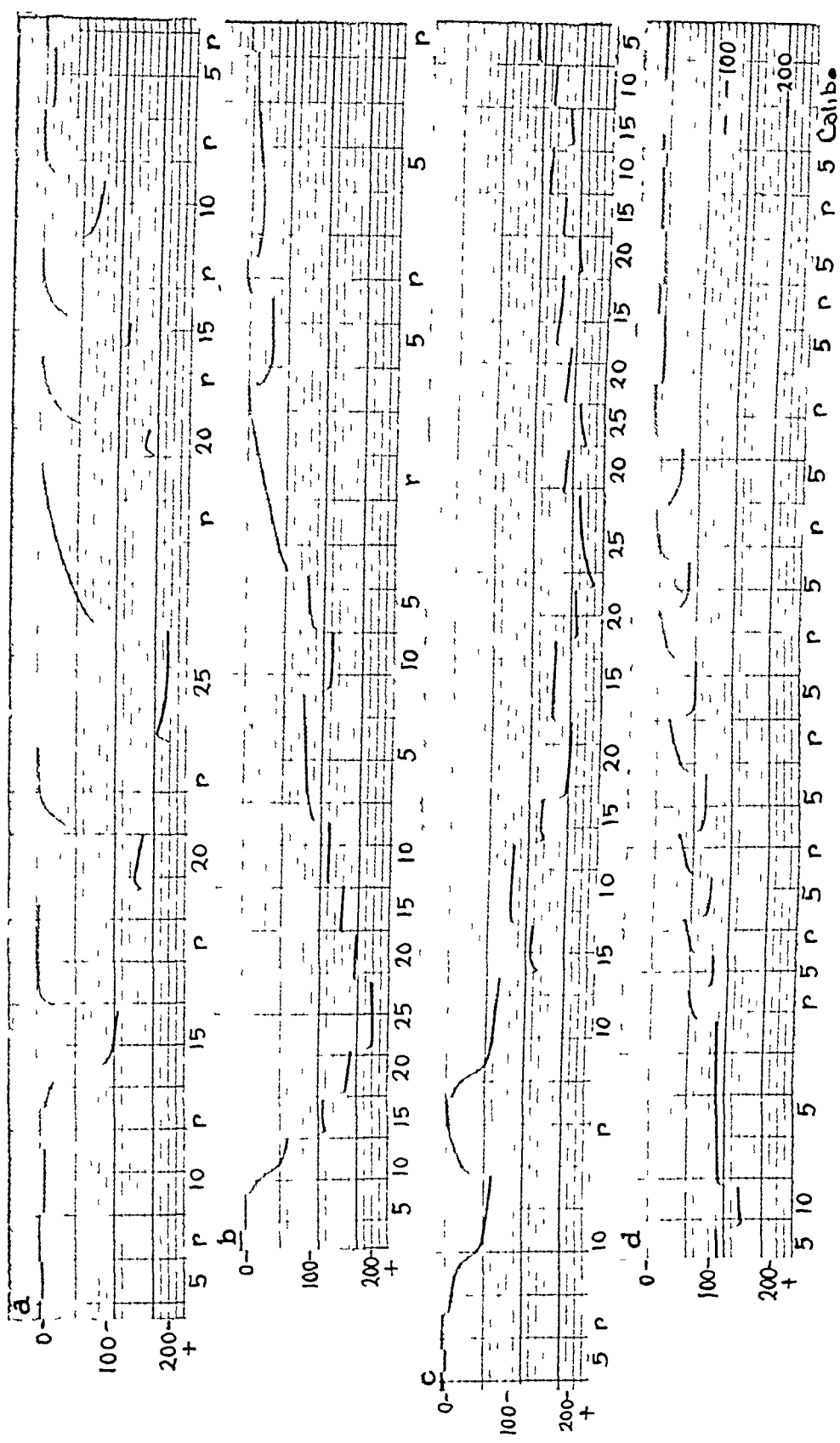


FIG 8

Further Characteristics of Regular Polarization

When the regular state is eventually regained, some of the transitional characteristics remain, especially the tendency for positivity to become more or less permanent following on current flow in either direction. This is seen in Fig 10, and becomes exaggerated when the cell is placed in acidified sea water (Fig 10 *e, f*). Here also the polarizations become much greater to outward than to inward currents, contrary to the usual condition. It is as if the *p.d.*, being already somewhat positive, can only be driven a small distance farther by inward current, while outward current has more leeway, being able to carry it to zero and some distance negative in addition. Otherwise, the regularity and symmetry of curves should be noted, with a good proportionality which falls off with increasing densities due to recessions from a higher value, at the cusp.

One further characteristic of polarizations in the regular state is shown in Fig 11, in which an attempt was made to determine whether the resistance in series with the cell had any influence upon the time course. Various extra resistances were introduced to bring the resistance in series with the cell from 40,000 ohms (capillary plus its equivalent balancing resistance) up to 100,000 and 200,000 ohms. Little if any change in the time curves results for the same current densities (produced of course by proportionally higher applied potentials).

FIG 8 Records illustrating characteristics of the variable state in *V. macrophysa* with delayed polarization and a threshold for polarization which is lower for decreasing currents than for increasing ones (hysteresis). Polarization was at first slight for 5 and 10 $\mu\text{a}/\text{cm}$ on Record *a* but 15 μa passing inward produced polarization with sigmoid course. Larger currents produce this more rapidly and add the characteristic cusp and recession which may be followed by a slower rise again. Decreasing currents maintain polarization down to 10 μa and even slightly at 5 μa . Record *b* shows a step wise increase and decrease of current without interruption of flow. Very regular increments and decrements of response obtain over part of the range. At 5 μa the positive *p.d.* begins to recede although 10 μa brings it back and after 4 seconds interruption during which only the residual current *r* passes 5 μa produce increasingly less effect. Records *c* and *d* show more of these steps, the last especially showing the slow disappearance of polarization on successive flows of 5 μa , until it becomes at last negligible. Currents are entirely inward across the protoplasm.

Sensitivity about 12.5 mv per division with values as indicated on each record and calibration on *d*. Time marks 1 second apart. Marks as in *f* and *g* Fig 1.

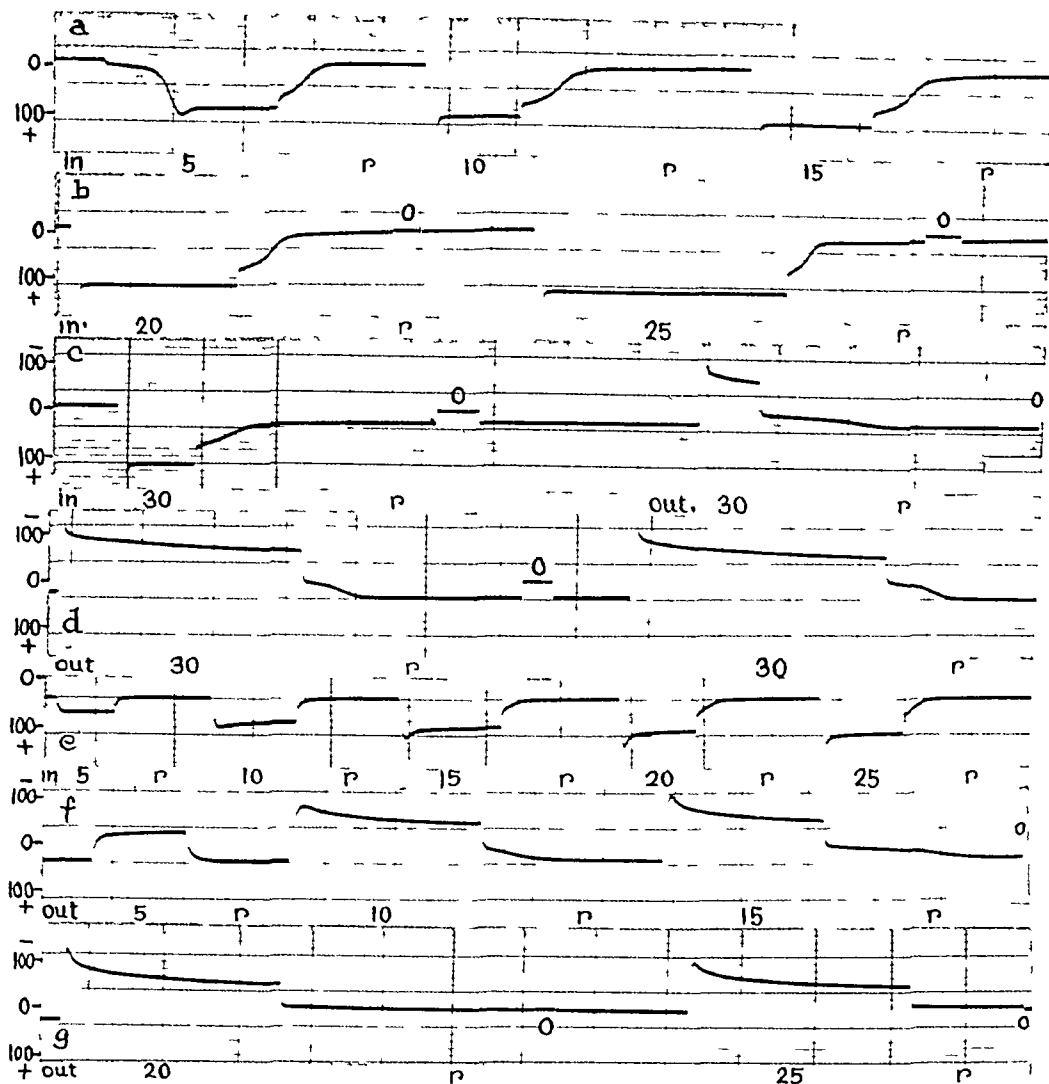


FIG 9 The production of lasting positive P D by current flow in *V. macrophysa*, and the further effects of current flow during positivity. The cell was an extremely hardy one which had lived impaled for 5 months, with frequent measurements (it finally died 7 weeks later). During much of this time it was in a transitional state between delayed and regular polarization, displaying small counter E M F 's to small currents in either direction, but also, superimposed on these, the characteristic sigmoid reversal curve, as shown in Record a. An increasing tendency is evident to linger at positive values after each inward flow, with an inflection around 60 mv positive, and a long slow approach to negativity. Finally, at $30 \mu\text{a}/\text{cm}^2$ the P D remains reversed (Record c) and returns to positivity even after an outward flow, although only after a lag at negative values (Record d). With the positive P D as a base, small inward currents are now passed (e), giving good polarizations, increasingly cusped at higher current densities. With outward currents (f), the polarizations are seen to be larger than with inward currents. Finally, after longer and longer lags at negative values (f) the P D remains again negative after outward flows (g).

Sensitivity about 13 mv per division, zero and 100 mv + and - being marked on each record (derived from calibrations not shown). Time marks 1 second apart. Residual current (r) when P D is negative, about $0.1 \mu\text{a}/\text{cm}^2$ (inward flow), when P D is positive, about $0.5 \mu\text{a}/\text{cm}^2$ (outward flow). Marks as in f and g, Fig 1.

The interpretation of this finding appears to be that a polarization capacity is responsible for the counter E M F s, since the charging time constant of a static capacity should be markedly increased by such increase of series resistance while that of polarization capacities is independent of it⁶

Experimental Production of Regular Polarization

It has been seen that regular polarization may be produced by (a) time, (b) the flow of sufficient inward current to condition the protoplasm. It remained to determine whether other treatments might be effective. A hint is given in Fig. 10, where acid sea water was found to exaggerate the normal regular characteristics to the extent of inducing greater positivity. Fig. 12 shows the effect of exposing a cell in the delayed state to sea water made slightly acid (pH 6.0) with HCl. As the record proceeds, the characteristics of delayed polarization pass over into those of regular polarization, the counter E M F s becoming larger and prompter for smaller currents until finally the exaggerated positivity and reversed polarizability of Fig. 12 c result after an hour's exposure.

These effects continue for some time after restoration of the cells to normal sea water, or even at higher pH, which suggests that internal, not external acidity is responsible for the maintenance of regular polarization. An attempt was therefore made to produce regular polarization by the application of various weak acids, such as acetic, butyric, etc., which are well known to penetrate cells readily. These were, however, scarcely more effective than HCl itself at the same pH, which suggests that it is the slow penetration of H ion (or possibly of CO₂ released from the carbonates of the sea water), which produces the effect. This point, however, merits further study, in view of the extraordinary effects of certain phenolic compounds (phenol, cresol, guaiacol) applied at Dr. Osterhout's suggestion. In low concentration (below 0.01 M) they promptly cause the P.D. to become positive, as in Fig. 13, and simultaneously induce a nearly regular polarization. These are, of course, extremely weak acids, and were found to reduce the pH of sea water and sap scarcely at all. Yet their effects persisted

⁶ Pierce W. M. *Phys. Rev.* 1928 36, 470

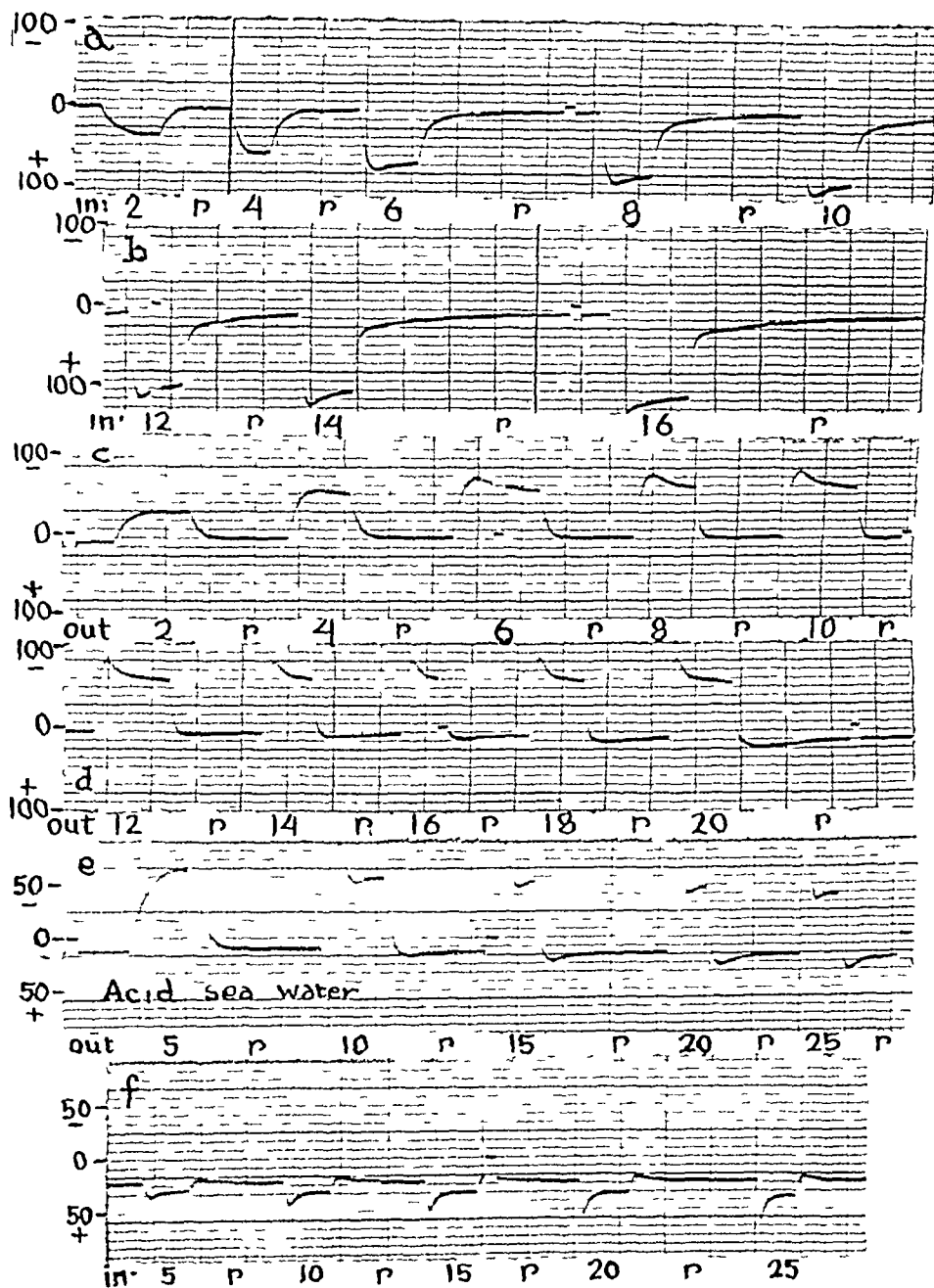


FIG 10

on replacing the cells in sea water, even of pH 10.3, for considerable periods, ammonia alone, as in Fig. 13, restoring delayed polarizability. Some specific chemical alteration is suggested.

(In these concentrations *p*-cresol is scarcely toxic and the effects may be produced in the same cell on several successive days without apparent injury.)

Effects of Ammonia

The application of ammonia was suggested by its very striking effects upon the P.D. of *Halicystis*, both alone⁷ and in combination with current flow.⁷ In effect, it turns that genus into a "*Valonia*" electrically, reversing its normal positive P.D. to negative values. Conversely, therefore, it seemed likely that it would restore negativity to *Valonia* when that organism had been made temporarily a "*Halicystis*," with positive P.D. This was indeed found to be the case: it promptly restored negativity, and with it delayed polarization, in cells

FIG. 10. Regular polarizations produced by current flow across the protoplasm of *V. macrophysa* impaled 7 weeks and thoroughly recovered from injuries so that it has attained the constant state of resistance. Current densities marked as in preceding figures. The lowest currents in either direction produce regular and proportional counter E.M.F.s which at higher densities, recede somewhat from the maximum value producing a cusp. The tendency for the steady bioelectric potential to become more positive as the result of current flow itself is here evidenced in Records *a* to *d*. As an extreme example of this tendency, showing the polarization characteristics which are produced upon a markedly positive bioelectric potential as a base, Records *e* and *f* are included; these were taken later with the same cell after it had been exposed for about 2 hours to acidified sea water (pH 6.0). The polarizations are prompt for both inward and outward currents but recession begins much sooner with inward currents, producing much less polarization (hence lower effective resistance) to inward than to outward currents. Note 'rebounds' or overshooting on depolarization.

Sensitivity in *a* to *d* about 11 mv. per division; increased in *e* and *f* to about 8 mv. per division. Zero and 100 or 50 mv. + and - indicated on each record with frequent (automatic) zero points inserted (sometimes interrupting the polarization record but not the current flow). Residual current (*r*) about 0.025 $\mu\text{a}/\text{cm}$ (inward) with the normal bioelectric potential increasing to about 0.015 $\mu\text{a}/\text{cm}^2$ (outward) with positive bioelectric potential. Time marks 1 second apart. Marks as in *f* and *g*, Fig. 1.

⁷ Report in preparation.

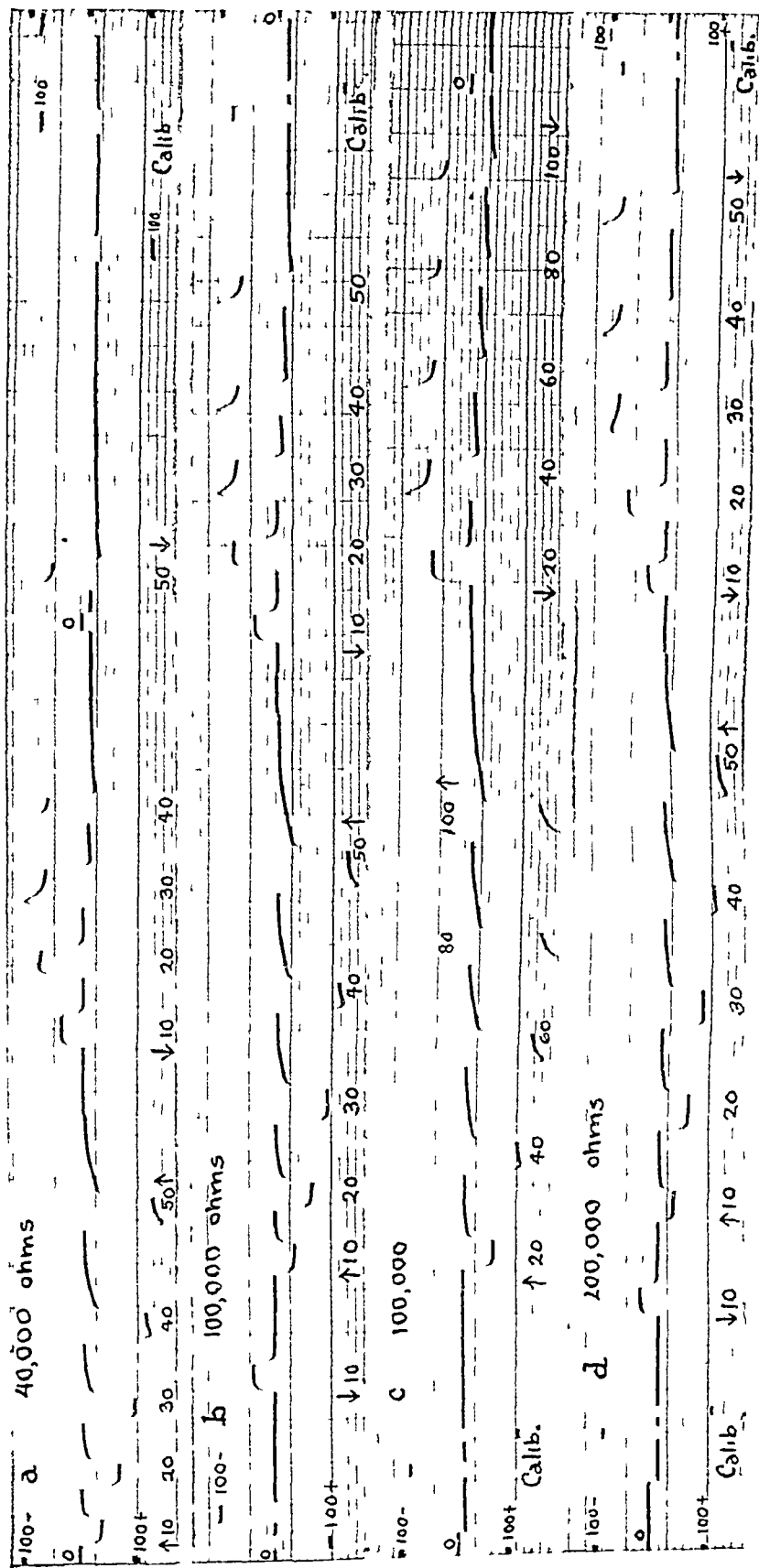


FIG 11

which had been rendered regular by *p* cresol (Fig 13), by acid (not shown), or had attained that state spontaneously (Fig 14). It also emphasizes the delayed state, reducing polarizations at sub threshold densities, increasing the threshold value, and exaggerating the sudden, all or none rise of positive rD , making the sigmoid curve almost rectangular in abruptness as shown in Fig 14. Furthermore, there is practically no persistence of the conditioning effect after cessation of inward current, polarizations to outward currents being practically abolished. On the other hand, during the actual flow of inward current, regular polarizations to small increments or decrements of that current are found in the middle range of densities (Fig 14) much as before, the inward current, during its flow, is able to counteract the ammonia effect.

The concentrations of ammonia which are effective in abolishing regular polarization and emphasizing the characteristics of delayed polarization vary somewhat from cell to cell apparently depending upon internal factors governing the permanence and extent of establishment of the regular state. Usually however about 0.005 M NH_4Cl in sea water of pH 8.0 was necessary to counteract regular responses in cells of *V. macrophysa* which had thoroughly attained that

FIG 11 The effect of series resistance upon the time relations of current flow. A cell of *V. macrophysa* in the constant state displaying immediate and proportional counter E M F s was measured first with the capillary resistance (20 000 ohms) just balanced in the adjacent bridge arm then with additional resistances introduced into each arm bringing the total resistances in series with the cell respectively to 40 000 ohms in Record *a*, 100 000 ohms in Records *b* and *c* and to 200 000 ohms in *d*. The time course of the counter E M F in speed of both rise and fall is found to be scarcely altered. This indicates that a polarization capacity known to have a time constant independent of series resistance is involved rather than a static capacity which should have its time constant increased 5 fold by the 5 fold increase of resistance.

The tendency for recessions to begin slightly earlier with outward than with inward currents is evident as well as an increasing tendency for the rD to remain at positive values. Both are characteristic of the constant state.

Currents indicated in $\mu A/cm^2$ as in previous figures upward arrows signifying inward currents downward arrows outward currents. Sensitivity is about 15 mv per division zero and 100 mv + and - being indicated on each record with calibrations on each. Residual current (not marked by r in this figure) depending on the resistances employed but not over 0.2 $\mu A/cm^2$. Time marks 1 second apart. Marks as in *f* and *g* Fig 1.

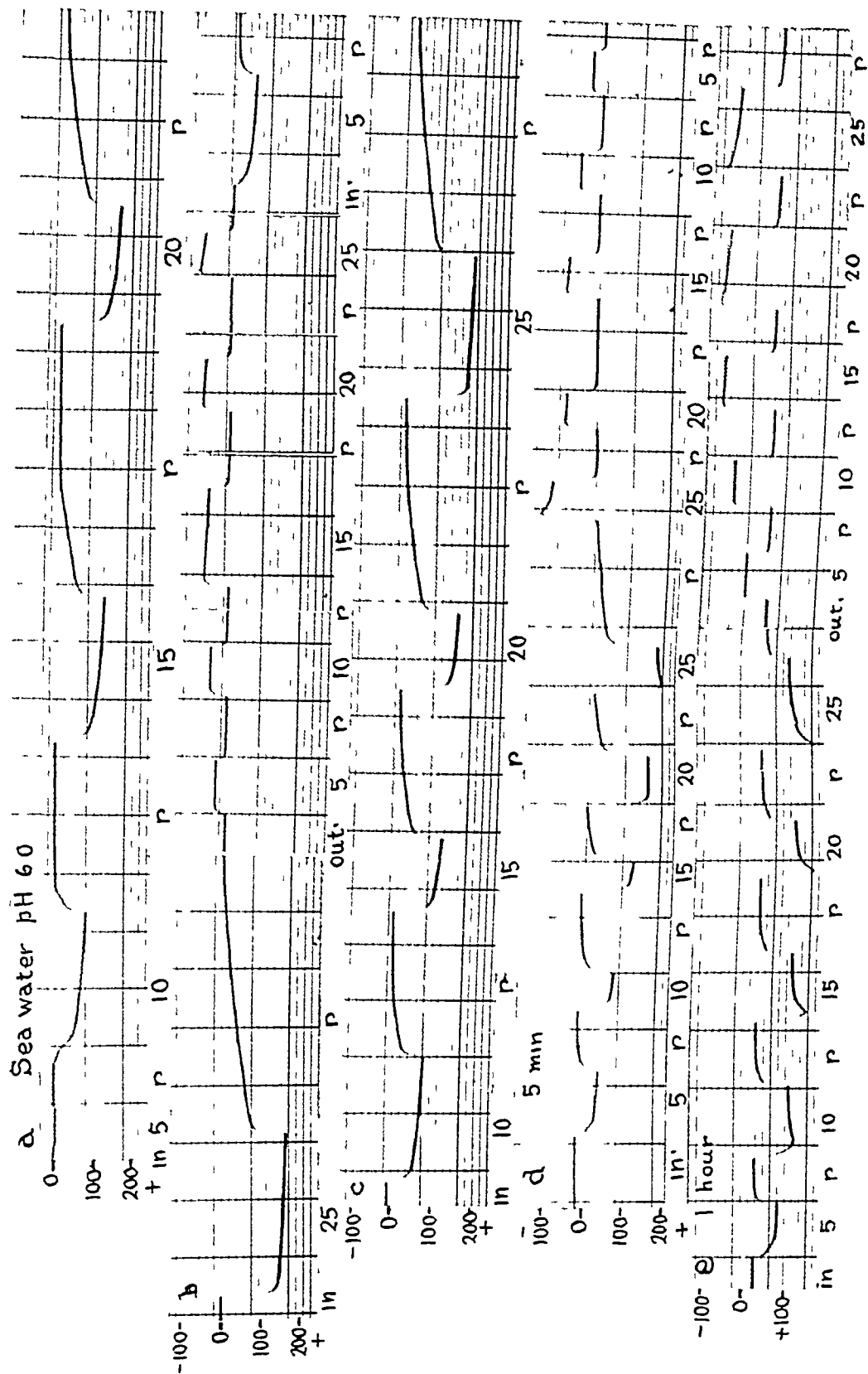


Fig 12

state 0.001 M and 0.002 M being in several cases ineffective over exposures of an hour or longer. (This value becomes reduced with increased pH of sea water, the effect apparently being dependent as in *Halicystis*⁴ upon undissociated NH_3 or NH_4OH .)

This experiment was not tried with *V. ventricosa* but based upon our observations⁸ a much smaller concentration would have been effective if indeed the cells stood the treatment at all since they usually break up into thousands of tiny cells (possibly this gives a hint as to the action of ammonia in incipient cases). *V. macrophysa* is however much more tolerant of ammonia as witnessed by the long life and extremely healthy appearance of cells exposed for several months to 0.005 M NH_4Cl in sea water.⁹ These same cells when tested electrically by us displayed a high resistance of regular type so that if the deconditioning had been produced by ammonia at all it had evidently passed off again and the cells had returned to regular polarizability. This is perhaps correlated with the fall of pH which occurred in their sap after a temporary rise due to entrance of ammonia⁹ and may be due to compensatory acid production by the cells (cf *Halicystis* also⁴).

The Effects of Potassium

Since KCl is present in high concentration (0.5 to 0.6 M) in the sap of *Valonia*, it was desirable to inquire whether the directional effects

FIG. 12. The production of regular polarization in *V. macrophysa* by increasing acidity. This is the same cell as employed in Fig. 7 where typical delayed polarization prevailed with practically no counter EMF developed by small currents in either direction across the protoplasm. The cell was then placed in sea water acidified with HCl to a pH of 6.0. Record *a* was taken immediately still showing the characteristics of delayed polarization although with a rather low inward threshold. By the time outward current was passed in *b* polarizations had become larger and prompter to small currents while remaining much the same as before to larger inward currents (*c*). In *d* taken 5 minutes later polarizations to outward current were approaching although not yet fully equaling those in inward current. Finally in *e* taken after 1 hour in acid sea water the P.D. has become permanently reversed (30 mv positive) and upon this as a base very regular polarizations are produced by the smallest currents nearly equal for inward and outward currents but because of the recessions with the former having a lower effective resistance to inward than to outward currents—the reverse of conditions in the delayed state.

Sensitivity about 18 mv per division zero and 100 mv + and - being indicated on each record. Time marks 1 second apart. Residual current (*r*) when P.D. negative about 0.05 $\mu\text{A}/\text{cm}$ (inward) when 30 mv positive (Record *c*) about 0.15 $\mu\text{A}/\text{cm}$ (outward). Marks as in *f* and *g* Fig. 1.

⁸ Unpublished results quoted by Steward F. C. in *Nature*, 1935 135 553.

⁹ Cooper W. C. Jr. and Osterhout W. J. V. *J. Gen. Physiol.* 1930-31 14 117.

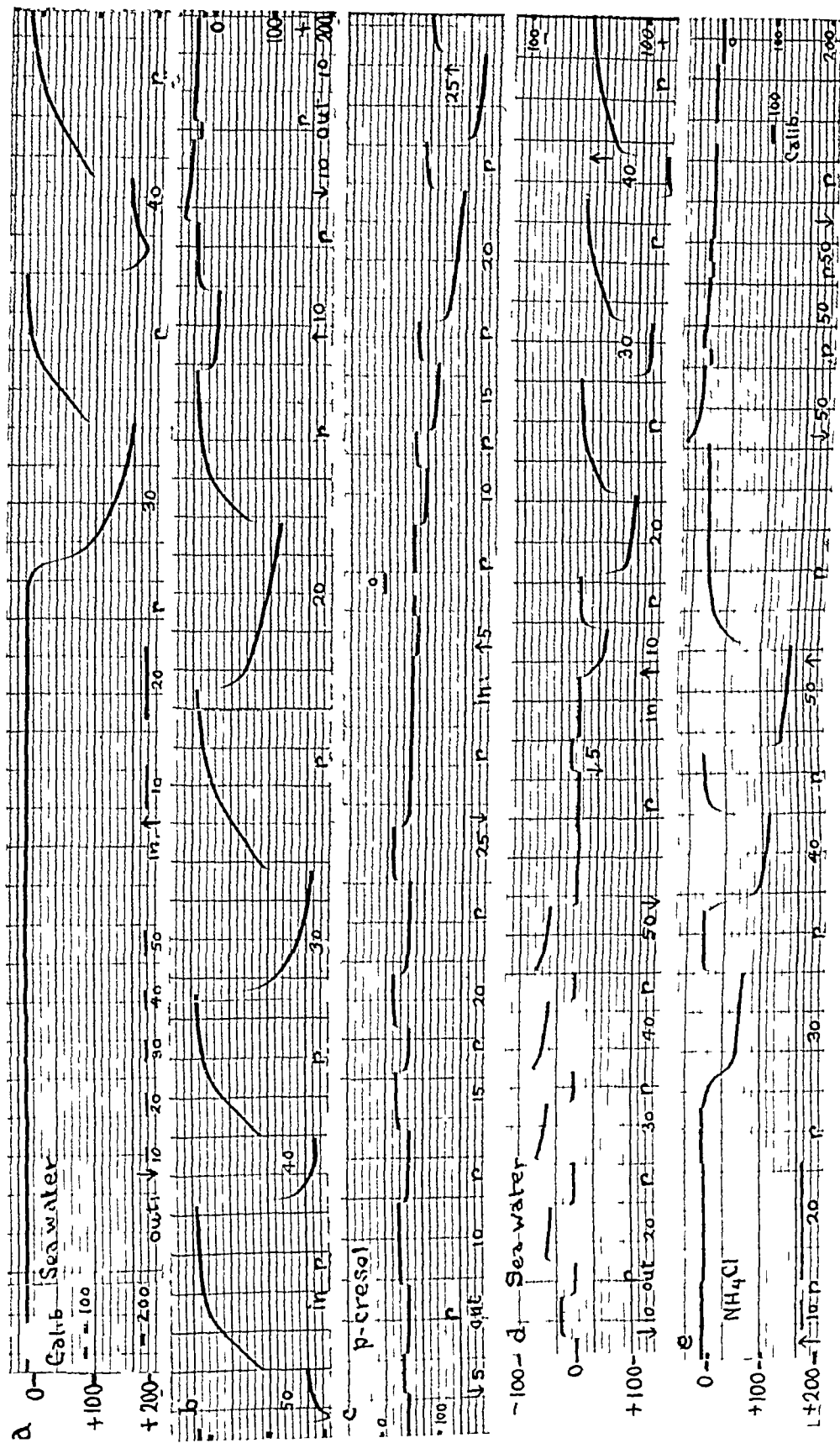


FIG 13

of current flow might be ascribed to the movement of potassium ions outward by outward flows, and inward by inward currents, enriching the protoplasm in the former case, and depleting it in the latter, of an ion assumed to have a high mobility therein¹⁰. The resistance and polarization would be expected to fall in the former case and to rise in the latter, while the persistence of polarization after inward flows (conditioning) might reflect the temporary depletion of potassium ions in the protoplasm, to be later regained, slowly by diffusion, or rapidly by flow of current outward from the sap. Similarly, the appearance of delayed polarization after impalement might be due to an injury which liberated KCl into the protoplasm from the vacuole, to be depleted again only by sufficient inward current (at the "threshold") to overcome the outward diffusion. Spontaneous recovery of regular polarization would on the other hand represent the loss of potassium from the protoplasm, either by diffusion outward or by the normal accumulatory processes of the cell.

FIG. 13. The production of regular polarization by *p* cresol, and its inhibition by ammonia. Records *a* and *b* show typical completely delayed polarization state in *V. macrophysa* impaled a few days. There is no counter EMF developed with outward currents up to $50 \mu\text{a}/\text{cm}^2$ and the threshold for inward currents is at $30 \mu\text{a}$ above which larger currents again produce small additional effect while smaller densities maintain polarization down to $20 \mu\text{a}$, and even slightly at $10 \mu\text{a}$ with a temporary response to $10 \mu\text{a}$ outward current. The cell was then placed in a dilute solution (0.01M) of *p* cresol in sea water. The PD immediately became about 50 mv positive as shown in Record *c* and polarizations began to appear to small currents in either direction, although still larger with inward than with outward currents. This condition persisted when the cell was replaced in ordinary sea water although the PD again became slightly negative. Record *d* was taken 10 minutes after restoration of sea water. Only when ammonia was added as in Record *e* did typical delayed polarization reappear.

Currents are in $\mu\text{a}/\text{cm}^2$ outward currents being designated by downward arrows, inward currents by upward arrows, the duration of currents being indicated where not made obvious by the counter EMF by bars above the figures. Sensitivity about 12.5 mv per division, zero and 100 and 200 mv values being placed on each record as derived from calibrations on *a* and *e*. Time marks 1 second apart. Residual current (*r*) about $0.1 \mu\text{a}/\text{cm}^2$ (inward) when PD is negative, $0.5 \mu\text{a}/\text{cm}^2$ (outward) when positive. Marks as in *f* and *g*. Fig. 1.

¹⁰ Damon E. B. *J. Gen. Physiol.*, 1932-33, 16, 375.

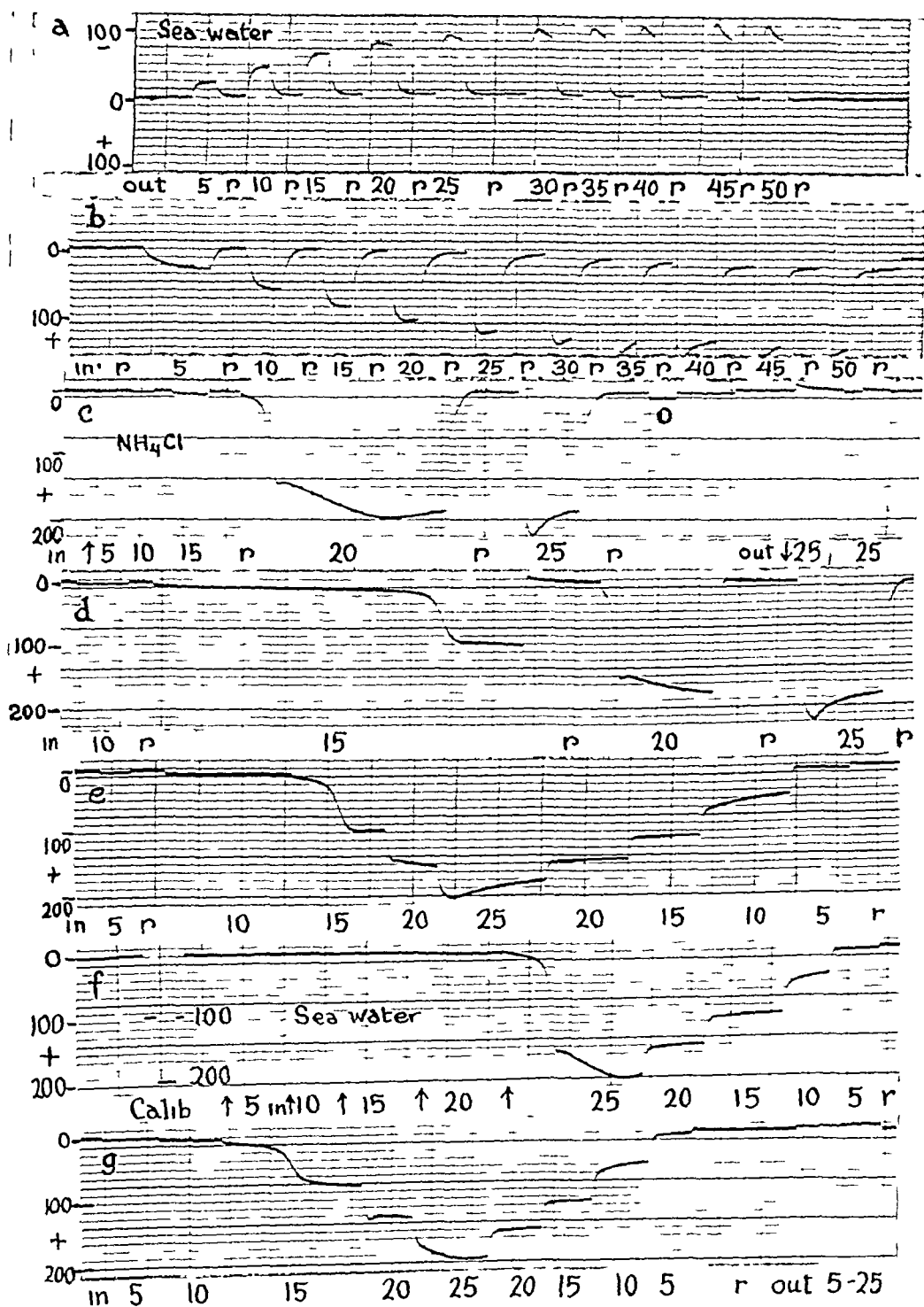


FIG 14

This hypothesis has been tentatively suggested in previous papers¹ and has been made by Osterhout the basis for a theory of the action current in *Nitella*¹¹

There appears, however, to be little basis for it in *Valonia*. Not only may the polarizability be controlled by quite other factors than potassium, (e.g. ammonia, acids, *p*-cresol) but the experimental modification of potassium concentrations seems to have little effect upon the polarizability, striking though the effects upon *i* D may be. Unfortunately it has not yet proved possible to perfuse the vacuole of *Valonia* with new solutions,¹ which might replace the normal KCl of the sap. But modification of the external solution is readily accomplished, the cells living well for some time in "potassium sea water" (van't Hoff artificial sea water with KCl entirely substituted for NaCl). The results are shown in Fig. 15, where the polarizability of a cell in

FIG. 14. Inhibition of regular polarization and production of delayed polarization in *V. macrophysa* by ammonia. A cell in the constant state displaying very regular and proportional counter E.M.F.s for low currents in either direction is shown in Records *a* and *b*. It was then placed for a few minutes in sea water of pH 8.0 containing 0.005 M NH_4Cl . Record *c* ensued: there was very little polarization to inward currents up to $20 \mu\text{A}/\text{cm}^2$ at which time a very abrupt sigmoid reversal curve was produced. $25 \mu\text{A}$ (and higher currents) produced only temporarily higher effects: the positive *i* D receding to about 180 mv after a cusp. Outward currents produce practically no counter E.M.F.s as shown in *c* even though following closely on inward flows: the conditioning effect is evidently short lived as shown also by the very sudden depolarization curves. In *d* the threshold to inward flow has fallen to $15 \mu\text{A}$, but with polarization occurring only after a very long lag. In *e* the effects of equal $5 \mu\text{A}$ increments from 10 to $25 \mu\text{A}$ and then $5 \mu\text{A}$ decrements down to residual flow (*r*) are shown.

The cell was then replaced in ordinary sea water without ammonia and Records *f* and *g* were taken after 10 minutes: the effects of ammonia are still present: there being little or no polarization to small currents and a threshold for inward flow at first $25 \mu\text{A}$ later 15 just as during ammonia treatment. Only after several hours did regular polarizability return. (It can be restored much more rapidly by acidified sea water.)

Sensitivity about 12 mv per division with 100 and 200 mv values being indicated on each record (calibration on *f*). Time marks 1 second apart. Residual current (*r*) about $0.2 \mu\text{A}/\text{cm}^2$ (inward). Marks as in *f* and *g*, Fig. 1.

¹¹ Osterhout W. J. V. *J. Gen. Physiol.* 1934-35 18 215

¹ Blinks L. R. *J. Gen. Physiol.* 1934-35 18 409

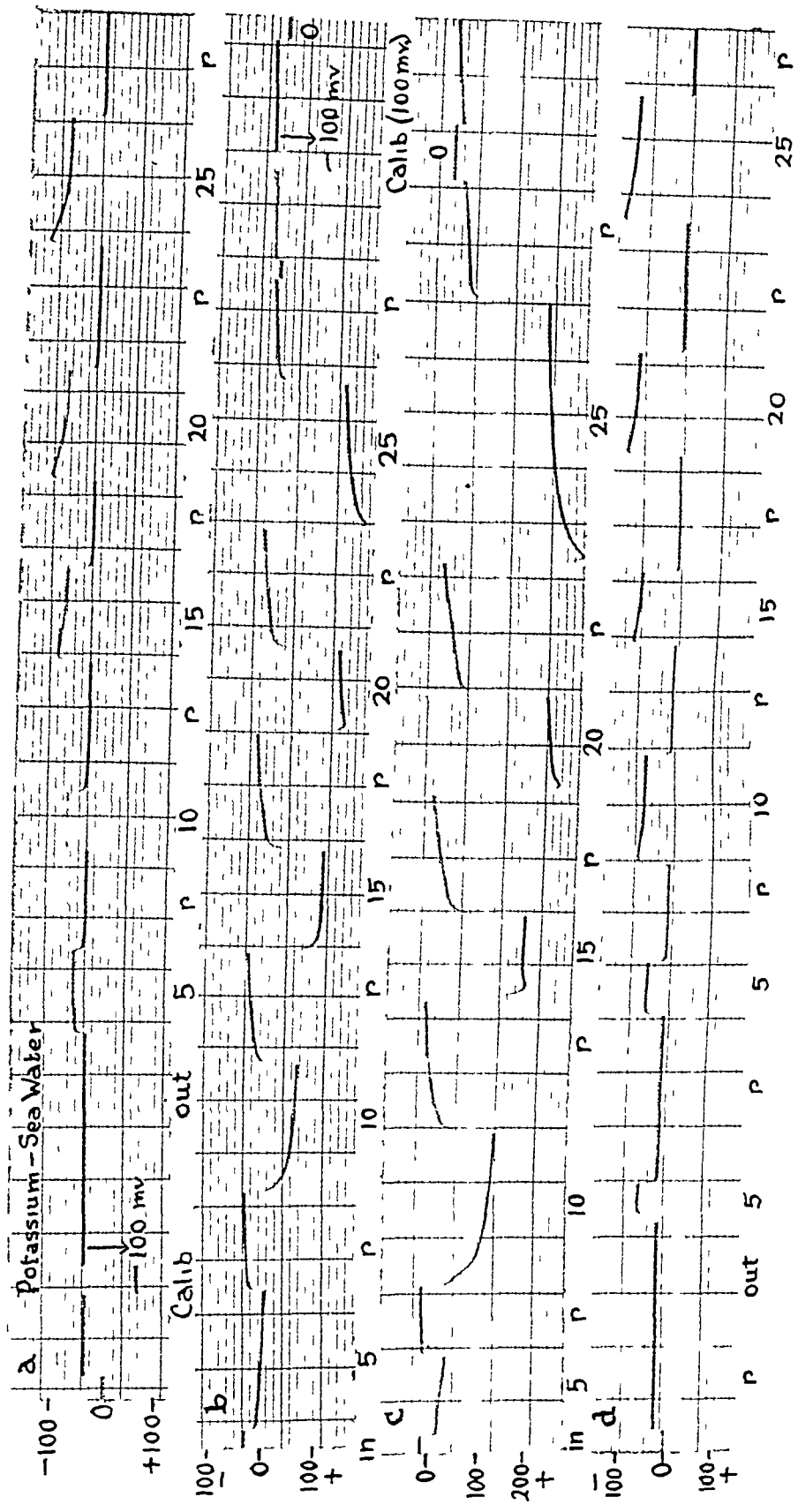


FIG 15

the transition state is scarcely distinguishable from that in ordinary sea water, despite the fact that potassium ions must now be moved from the outer solution into the protoplasm by inward currents as rapidly as they are moved from it into the vacuole. Depletion of these ions by inward flow can thus scarcely account for the polarizations produced.

Similar indifference of the protoplasm in its regular state is displayed to potassium sea water, polarizations being as large and as prompt as in normal sea water, showing that KCl does not immediately enter the protoplasm and produce the delayed state, and that the resistance to this salt remains about as high as to any other.

Practically the only difference sometimes observable is a rather more abrupt rise of positive $P.D.$ at the threshold or above, somewhat evident in Fig. 15. This corresponds to the ammonia effect (Fig. 14), although it is much less pronounced. It would indeed be expected if potassium entered as KOH corresponding to the entrance of NH_3 or NH_4OH . Its entrance in this fashion, however, is evidently so slow as to produce little effect upon the threshold, etc.

(These statements hold for exposures of the cells to potassium rich solutions for several hours. Exposures of a day or longer are eventually effective in that the cells display greatly reduced polarization. While this might be regarded as evidence that potassium ions have at last begun to permeate the protoplasm, and to produce low polarization by their high mobility, the magnitude of times concerned in this effect is quite different from those met with in the polarization phe-

FIG. 15 Polarization in the presence of high potassium concentration. Shortly before the start of Record *a* *V. macrophysa* had been placed in potassium sea water (artificial sea water according to van t Hoff but with KCl substituted entirely for NaCl). The $P.D.$ had become as usual more negative. Good polarizations persisted with currents in either direction although somewhat larger with inward currents (Record *b*). After 20 minutes exposure to this solution, during which the $P.D.$ fell somewhat, even larger polarizations were obtained as shown on Records *c* and *d*. The very rapid rise and fall of polarization curves are characteristic of this condition.

Sensitivity about 16 mv per division. Zero and 100 mv + and - are marked on each record with calibrations on *a* and *b*. Time marks 1 second apart. Currents in or out, as marked in $\mu a/cm$ of cell surface. Residual current (r) about $0.2 \mu a/cm^2$ (inward) in Record *a*, falling to about $0.08 \mu a/cm$ in *d*.

nomena that it aims to explain, and it seems to be due rather to a profound injury¹³ from which there is no recovery on restoration to sea water)

DISCUSSION

Since the movement of potassium ions cannot apparently account for the phenomena of delayed polarization in *Valonia*, to what may the effects of inward current flow be attributed? It seems reasonable to assume that regular polarizations are due to the maintenance of an intact surface, across which ionic mobilities, partition coefficients, etc., govern the production of counter E M F. This surface may apparently be profoundly altered in some way, so that polarizations disappear, without killing or seriously injuring the cell, since it lives for some time in the state of delayed polarization. In this state there is apparently free ionic transfer across the surface, the effective resistance of the protoplasm being practically nil, and its P D low and negative. This remains true for large outward currents, but sufficient inward current suddenly restores polarizability, along with a strongly positive P D. Since polarizability persists for a while thereafter to smaller currents, we may assume that the current flow has actually restored the surface of the protoplasm, or at least those of its properties governing polarization.

Furthermore, since this effect of inward current is duplicated by certain chemical treatments such as acidified sea water and various phenolic acids, which produce a positive P D and restore polarizability, the conclusion is suggested that inward current produces its "restorative" or conditioning effects by an increase of acidity, or an acid-like action, upon some structure of the protoplasm. Conversely since both outward currents and penetrating bases like ammonia tend to destroy polarizability and produce a negative P D, we may postulate that they alter the surface (or its electrical properties) by an increase of alkalinity at some critical point. A possible mechanism would be a saponifying action upon lipid constituents by bases or outward currents, counteracted by esterification and reformation of an oily or fatty acid film under the influence of acidic substances or of inward currents.

It is both theoretically possible (through the high mobility of H ion)

¹³ Osterhout W J V, *J Gen Physiol*, 1924-25 7, 561

and experimentally demonstrated¹⁴ that current flow can produce such acidity changes (membrane electrolysis) at various interfaces such as gels and membranes. While the evidence upon which similar effects were postulated in living cells appears to have been erroneous,¹⁵ it is still quite conceivable that it occurs, even though not so visibly as claimed. We are strongly inclined to think that it must occur, on the basis of the *Valonia* phenomena here described, and on even better evidence (additive effects of ammonia and current flow) in *Halicystis*.⁷ It will be discussed further elsewhere. However, the ascription of common causes to common effects is dangerous, and it may only be that various agents can act upon the same function (polarizability) in an all or none manner, both outward currents and weak bases destroying it, both inward currents and weak acids restoring it, but each acting through a different mechanism.

Comparison with Ag AgCl Electrodes

Although they have certainly nothing but a formal resemblance to the curves obtained with *Valonia*, the accompanying string galvanometer records (Fig. 16) of current flow with a silver-silver chloride electrode of small area may be suggestive of what happens when a reversible ("non-polarizable") electrode becomes irreversible or polarizing by the passage of large currents (here reducing the AgCl to Ag). The slight polarization to small "inward" currents (electrode negative) followed by a delayed polarization with sigmoid curve at the threshold density, the relatively quicker polarizations with succeeding current flows and larger densities and the restoration of non-polarizability by outward flow (electrode positive), are all reminiscent of the *Valonia* records.

Grateful acknowledgment is made to the Carnegie Institution of Washington for opportunities to study at its Dry Tortugas Laboratory.

SUMMARY

The effect of direct current flow upon the potential difference across the protoplasm of impaled *Valonia* cells was studied. Current density

¹⁴ Bethe, A., and Toropoff, T. *Z. phys. Chem.* 1914, 88, 686; 1915, 89, 591.

¹⁵ Blinks, L. R. *Proc. Soc. Exp. Biol. and Med.* 1932, 39, 1186. These qualitative observations of the author have been verified by Mr. R. D. Rhodes in the Stanford laboratories by objective photographic and spectrographic records which are being prepared for publication.

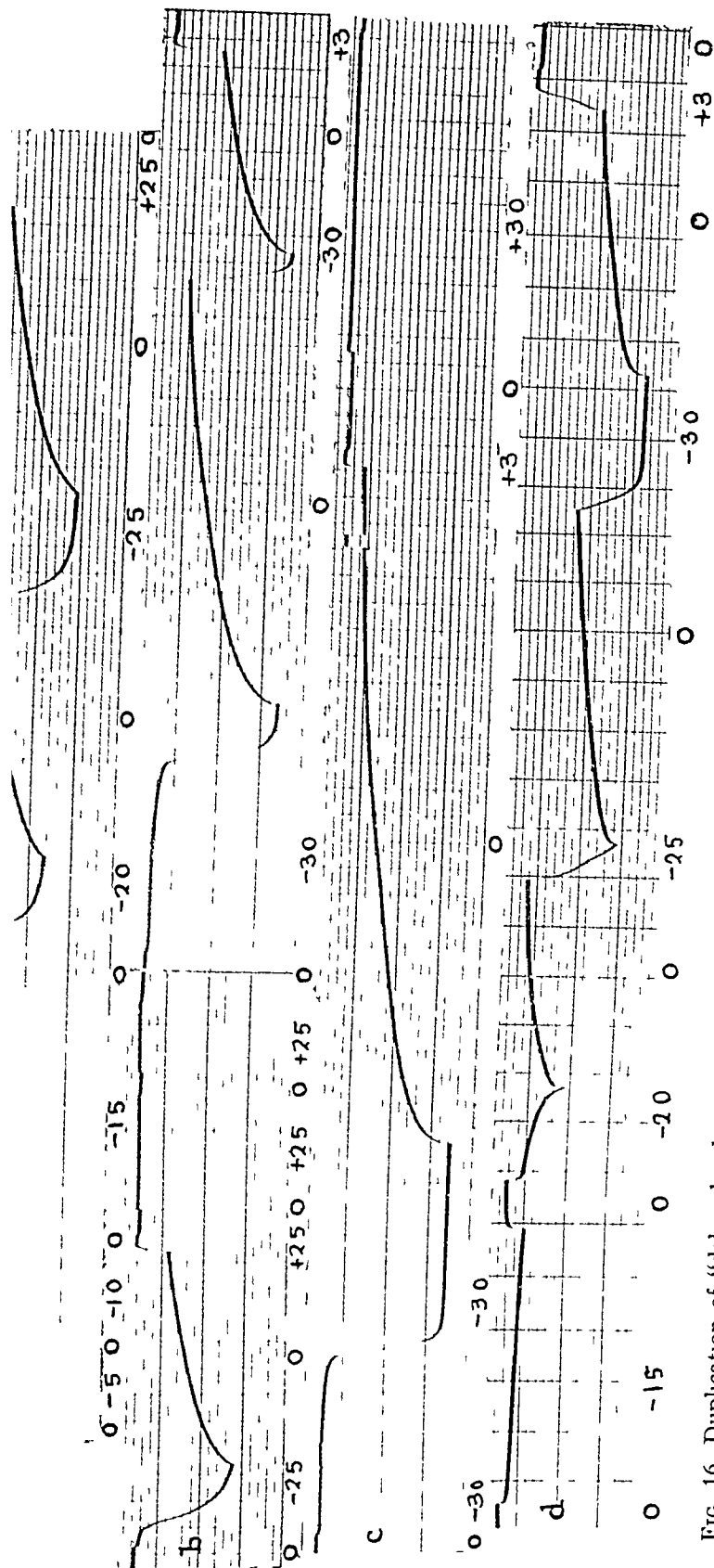


Fig 16 Duplication of "delayed polarization" effects with electrode model Two Ag-AgCl electrodes, one of small and one of large area were subjected to current flow in the bridge circuit used for *Valonia* The large electrode had sufficient surface to remain non-polarizable with all current densities employed, the smaller was non-polarizable to lower current densities, but became polarized with a sigmoid curve of counter E v r at a critical threshold density of inward current (electrode negative, attracting positive current) Long slow depolarizations follow cessation of current flow, with a tendency for the electrode to retain a positive charge Passage of outward current with the electrode made positive, then rapidly reduced this charge and made the electrode again non-polarizable (by deposition of AgCl), both to continued outward current, and again to inward current of low density This resembles the conditioning and deconditioning in *Valonia* by inward and outward currents

The actual sensitivities were not recorded, nor the current densities The figures stand for applied potential in volts positive or negative, no current passing when 0 is indicated Time marks 1 second apart

and direction were controlled in a bridge which balanced the ohmic resistances, leaving the changes (increase, decrease, or reversal) of the small, normally negative, bioelectric potential to be recorded continuously, before, during, and after current flow, with a string galvanometer connected into a vacuum tube detector circuit.

Two chief states of response were distinguished.

State A—Regular polarization, which begins to build up the instant current starts to flow, the counter $E M F$ increasing most rapidly at that moment, then more and more slowly, and finally reaching a constant value within 1 second or less. The magnitude of counter $E M F$ is proportional to the current density with small currents flowing in either direction across the protoplasm, but falls off at higher density, giving a cusp with recession to lower values, this recession occurs with slightly lower currents outward than inward. Otherwise the curves are much the same for inward and outward currents, for different densities, for charge and discharge and for successive current flows. There is a slight tendency for the bioelectric potential to become temporarily positive following these current flows.

Records in the regular state (*State A*) show very little effect of increased series resistance on the time constant of counter $E M F$. This seems to indicate that a polarization rather than a static capacity is involved.

State B—Delayed and non proportional polarization in which there is no counter $E M F$ developed with small currents in either direction across the protoplasm, nor with very large outward currents. But with inward currents a threshold density is reached at which a counter $E M F$ rather suddenly develops, with a sigmoid curve rising to high positive values (200 mv or more). There is sometimes a cusp after which the $P D$ remains strongly positive as long as the current flows. It falls off again to negative values on cessation of current flow more rapidly after short flows, more slowly after longer ones. The curves of charge are usually quite different in shape from those of discharge. Successive current flows of threshold density in rapid succession produce quicker and quicker polarizations, the inflection of the curve often becoming smoothed away. After long interruptions, however, the sigmoid curve reappears. Larger inward currents produce relatively little additional positive $P D$ smaller ones on the other hand,

if following soon after, have a greatly increased effectiveness, the threshold for polarization falling considerably. The effect dies away, however, with very small inward currents, even as they continue to flow. Over a medium range of densities, small increments or decrements of continuing inward current produce almost as regular polarizations as in State A.

Temporary polarization occurs with outward currents following soon after the threshold inward currents, but the very flow of outward current tends to destroy this, and to decondition the protoplasm, again raising the threshold, for succeeding inward flows.

State A is characteristic of a few freshly gathered cells and of most of those which have recovered from injuries of collecting, cleaning, and separating. It persists a short time after such cells are impaled, but usually changes over to State B for a considerable period thereafter.

Eventually there is a reappearance of regular polarization, in the transition there is a marked tendency for positive P/D to be produced after current flow, and during this the polarizations to outward currents may become much larger than those to inward currents. In this it resembles the effects of acidified sea water, and of certain phenolic compounds, *e g* *p*-cresol, which produce State A in cells previously in State B. Ammonia on the other hand counteracts these effects, producing delayed polarization to an exaggerated extent.

Large polarizations persist when the cells are exposed to potassium-rich solutions, showing it is not the motion of potassium ions (*e g* from the sap) which accounts for the loss or restoration of polarization.

It is suggested that inward currents restore a protoplasmic surface responsible for polarization by increasing acidity, while outward currents alter it by increasing alkalinity. Possibly this is by esterification or saponification respectively of a fatty film.

For comparison, records of delayed polarization in silver-silver chloride electrodes are included.

THE POLARIZATION CAPACITY AND RESISTANCE OF VALONIA

I ALTERNATING CURRENT MEASUREMENTS

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Previous reports¹⁻⁴ have shown that the effective direct current resistance of *Valonia* protoplasm is associated with, and is probably largely due to the building up of a counter E M F during the flow of current. This opposes the applied E M F and decreases the current flow below its initially larger value. The counter E M F is not only large in magnitude (up to 200 or 300 mv), but takes an appreciable time to appear or disappear ($\frac{1}{2}$ to several seconds). Such slow charge and discharge can be ascribed, if desired, to a large electric capacity of the protoplasm.

A capacity could result from either of two types of protoplasmic structure. (1) There could be a thin lipid or other insulating film at one or both surfaces of the protoplasm, with zero, or low and equal, permeability to ions of both charges. (2) There could be one or more phase boundaries (or other non uniformities) across which ions of one sign had a higher mobility than those of the other.

The first type should act like a condenser whose plates are the solutions in contact with the dielectric film, with a capacity dependent on its dimensions and its dielectric constant. The second type should act like a polarizing electrode by setting up a differential concentration of ions at the boundaries.

By analogy with non living systems, these two types of capacity

¹ Blinks, L R, *J Gen Physiol*, 1929-30, 13, 361

² Blinks L R, *J Gen Physiol*, 1929-30, 13, 793

³ Blinks, L R, *J Gen Physiol*, 1930-31, 14, 139

⁴ Blinks, L R., *J Gen Physiol*, 1935-36, 19, 633

should be distinguishable by their time relations, the first, or "static" type having a capacity which is constant with time during current flow, the second, or "polarization" type having an effective capacity which is not constant, but increases with time during current flow⁵

These differences are reflected in both the direct and alternating current behavior of the circuits. The present paper will present evidence obtained by alternating current measurements. As a background for these, however, it is desirable to indicate some of the direct current results which have been presented elsewhere.

It was found, for example that many of the direct current phenomena could not readily be referred to the charge and discharge of a static condenser. This is most strikingly seen in the so called "delayed polarization" stage. Here the course of potential change is far from a regular exponential curve. A counter $\epsilon m f$ may even be entirely lacking at low current densities, to appear only above a critical threshold density. It then builds up in a sigmoid curve with a slow start, an inflection, and a more rapid rise, the latter often followed by a sharp cusp, and a recession to a final constant value. This final value is not proportional to the applied potential, but has a low or zero value below the threshold density, increases more linearly above this, then again falls off from proportionality at higher densities. The discharge curve is also not regular, but often shows an inflection or even a marked delay at intermediate levels. The time course of counter $\epsilon m f$ is different at different current densities, increasing in speed with larger currents, it also changes form with successive flows of the same current density, becoming again more rapid with later flows. It is usually different for charge and discharge, and notably so for currents in opposite directions across the protoplasm. Finally its speed is not appreciably decreased by the introduction of higher resistances in series with the protoplasm, as would be the case with a condenser.

This brief summary indicates the extreme divergence of these phenomena from the behavior of a static condenser, which should show strictly exponential curves of identical form for charge and discharge, for successive exposures, for currents in either direction, and—except for differing magnitudes—for currents of any density. In the latter case the counter $\epsilon m f$ should be proportional to the applied potential. The phenomena must therefore be referred to some sort of polarization, but even this is of a curious, delayed sort, with curves which sometimes continue to change for several seconds. Obviously an incomplete picture of such behavior would be obtained with the usual alternating current frequencies.

There are, however, much more regular curves displayed by *Valonia* under some circumstances. These occur, for example, as part of the picture in the delayed and variable polarizations just discussed. Over the middle range of

⁵ Fricke, H., *Phil Mag*, 1932, 14, series 7, 310

current densities, where the magnitude of the counter $\epsilon m f$ is nearly proportional to the applied potential, the curves are also much more regular, both for charge and discharge of the total current, and for smaller increments or decrements of a continued polarizing inward current. It is as if the polarizing current had "conditioned" the protoplasm to a more regular, and less delayed state. (Analogous behavior is shown by Ag/AgCl electrodes which may be changed from a non-polarizing to a polarizing condition by the continued flow of direct current, with marked resemblance to the *Valonia* curves)⁴

Regular curves are also found in cells which have reached a "constant" resistance level, either in nature, by sojourn in the laboratory, or by experimental treatment such as exposure to acidified sea water or to certain phenolic compounds⁴. The constant state is characterized by an apparent resistance which is constant over a wide range of direct current density, this is the same as saying that the counter $\epsilon m f$ is proportional to the applied potential. Not only this, but the curves are much more regular, starting instantly at make or break of current, and asymptotically approaching their final value. They are approximately symmetrical as to charge and discharge and identical with successive current flows, in either direction across the protoplasm. They are also of nearly the same form differing only in magnitude, with currents of increasing density (except at very large values where a recession, possibly due to breakdown, occurs after the highest point of the curve).

In the constant state the curves suggest that a true capacity is involved. The properties mentioned, however, are alike characteristic of a static condenser or of a polarizing, irreversible electrode (e.g. platinum in KCl). The question as to which kind of capacity is displayed must therefore be answered by determining whether the capacity is constant or changing with time during current flow.

Several methods are available for investigating this. One is inspection of the counter $\epsilon m f$ curves obtained on direct current records. If they adhere to an exponential time course, the capacity is constant and static, if not, the deviations should tell something of the polarization characteristics. This method will be applied in another paper.

A second method is direct comparison of the cell's reactance in a bridge circuit with capacities of known type. These may be static condensers of constant capacity, or electrodes displaying typical polarization capacity. The latter are difficult to adjust to a given capacity, but a few exploratory experiments have indicated that it is sometimes possible to balance the cell's reactance fairly closely with such polarization capacities. More convenient is a variable mica

condenser which more or less entirely compensates the cell's curves, depending on how closely these follow an exponential course. Deviations from the latter will actuate the detector, giving in direct current a residual deflection which is due to the progressive change of the cell's capacity with time, or in alternating current a sound in the telephone at all but one frequency. The direct current residuals are closely related to analysis of the complete time curves, and will be considered in connection with these elsewhere. The alternating current measurements are taken up below.

It may be pointed out that the A C measurements complement the D C ones in satisfactory fashion, since they cover the earlier parts of the time curves (below the first $1/100$ second) where the period of the galvanometer might introduce faulty recording. Conversely, the D C records become reliable on the later parts of the curves (up to $\frac{1}{2}$ second or more) where it is cumbersome or impossible to generate or measure A C of sufficiently low frequency to be applicable. The two methods together should thus give a more complete description of the whole time course due to capacity.

It might also be remarked that in the case of *Valonia* it is desirable or even essential that the cells be tested by D C recording immediately before and after the A C runs, not only to establish the value of their effective resistance which is used as a shunt in the balancing arm, but also to make sure that the cells initially show and remain in the constant state where immediate and regular polarizations are being displayed, and where alone the A C measurements would be significant. This was always done in the present studies. In other cells, where the polarization characteristics are not so variable, this precaution is probably not so necessary.

Apparatus

A conventional Kohlrausch (A C Wheatstone) bridge was used.

The methods of measuring resistances and capacities in such a circuit are sufficiently standard⁶ that they need not be elaborated here. Many of the difficulties met with at higher (radio) frequency measurements were avoided by the use of rather low frequencies (mostly in the audio-range). These were possible

⁶ Hague, B., *Alternating current bridge methods*, London, Sir Isaac Pitman & Sons, 1923.

because of the extremely high actual capacities displayed by *Valonia*, resulting from but one or two protoplasmic layers across which current flowed, instead of hundreds or thousands in series, as in most biological material such as tissues or cell suspensions. The impedance accordingly falls off at much lower frequencies to a minimum value, as shown in Fig. 1.

In spite of reduced trouble with ground and inter arm admittances at these lower frequencies, care was taken to keep the bridge symmetrical, and partially shielded. Shielded elements included the cables leading to and from the bridge, the input and output transformers, and the equal ratio arms (of 100 or 1000 ohms each General Radio Co. resistances). A Wagner ground circuit with resistive and capacitive balance kept the detector at ground potential.

General Radio Co. decade resistance boxes, Ayrton Perry wound to have small reactance were used as variable standards. A Leeds and Northrup 3-decade mica condenser, reading from 0.001 to 1.00 mfd. in 0.001 mfd. steps, was used as the capacity standard, supplemented by a General Radio Co. variable air condenser, and two fixed mica condensers (Dubilier) of high quality, of 1 mfd. each.

These standards, and the apparatus holding the cells, were not shielded. Measurements of fixed resistances and capacities substituted in the electrical position of the cells (i.e., in series with the actual electrodes and salt bridges employed) showed nearly constant and correct values over the frequency range employed. Similar constancy of resistance, and essential absence of capacity effects were found with the electrolytic system used for contact with the cells (salt bridges such as capillaries, glass tubes, and agar blocks). The calomel or lead-lead chloride electrodes of large surface were practically non-polarizable (i.e., had such a large series capacity that the much smaller ones in the cells entirely governed the readings).

Some remarks concerning the current characteristics are of interest as these have possible biological effects. A General Radio Co. Type 377 audio frequency oscillator was employed (about 1 meter from the bridge) to generate the alternating current, continuously variable in frequency from 60 cycles up to 80,000 cycles. This uses an oscillating tube (201 A or 112 A) feeding through a variable resistance coupling to an output tube (112 A) which in turn, feeds out through a condenser. The output tube guards against influences of the bridge impedance upon the frequency or wave form of the oscillator.

The oscillator tube was worked with the lowest possible feedback into the grid which would maintain (not initiate) oscillation. This kept down harmonics to their lowest value. While the latter were still appreciable, it was usually possible to distinguish the more powerful fundamental in the telephone, and to balance the bridge to silence with respect to this, neglecting the higher overtones. No attempt was made to determine what proportion of the total current through the cells was carried at these higher frequencies, according to available data for the oscillator it amounts to a few per cent. It seems doubtful that this could influence the capacity measured at the fundamental frequency but it may be a

source of disturbance. Also, passage of current through the cell might actually produce harmonics (suggestion of Dr K S Cole⁷)

The total current through the cells was determined in a few typical cases with a heater-thermocouple or rectifier type ammeter, and was found to be from 10 to 50 microamperes, a range found in D C to be non-injurious⁴. That this was sufficiently low to avoid injury and breakdown was also shown by the unchanged capacity and resistance readings obtained when the current was increased two- or threefold. Very large currents, however, had an effect, curiously enough increasing the apparent capacity. This could result from dielectric breakdown at the peak potentials, uncorrected by a lowered shunt resistance in the balance arm. It is an effect which might give spurious capacity changes in A C measurements. By keeping the currents as low as possible for audibility of the signals, this is probably avoided.

The detector employed a 2- or 3-stage audio-amplifier, to bring the weak signals up to audibility in the telephone. With higher frequencies (above 10,000 cycles) where audibility is low, a heterodyne oscillator fed into the detector circuit to give beats of audible frequency.

A double run, beginning with the lowest frequency, passing up to the highest, and then down again was usually made with each cell. At frequencies above 20,000 cycles, adjustments of capacity became rather meaningless, since the reactance of the large capacity involved is already so low as to give silence over a wide range of setting (with proper resistance values). Readings were therefore not usually carried to higher frequencies.

The two species of *Valonia* studied have given consistent results, when care was taken that they were in the constant resistance state. *V. macrophysa* has been chiefly employed, both in Bermuda and in New York, *V. ventricosa*, in Florida (Tortugas) and New York.

EXPERIMENTAL

Measurements were first made with intact cells, electrical contact being obtained at each end of the cell through agar blocks saturated with sea water. The arrangement of these was the same as for the D C measurements earlier described¹. This restricts the leakage or shunt conductance around the cell to its lowest possible limits, namely that of the cell wall itself, imbibed with sea water. Most of the current must therefore pass through the protoplasm, with consequent emphasis on the large capacitative reactance of that path. The resulting change of impedance at low frequencies is clearly shown in Fig 1, which plots the results of balancing the cell against the simple circuit

⁷ Personal communication. See also Lane, C T, McCulloch, W S, Prescott, C H, and Dusser de Barenne, J G, *Am J Physiol*, 1935, 113, 85.

of a resistance in parallel with a capacity, shown on the same figure. This is the conventional arrangement for balancing electrolytic conductivity bridges, and may be called Circuit A.

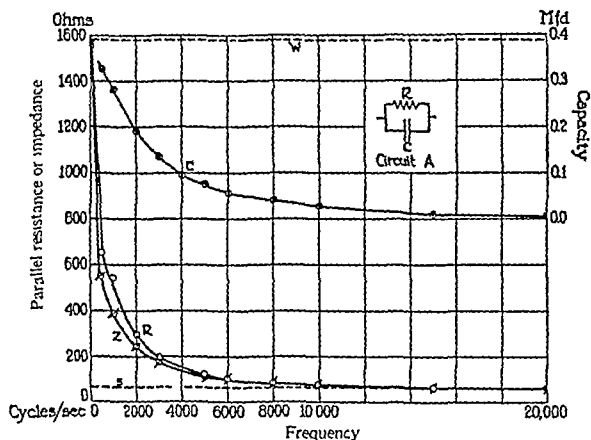


FIG. 1 Alternating current characteristics of a *Valonia* cell, held between external contacts (bridging an air gap between agar blocks) when balanced against the simple parallel Circuit A as shown. Both the equivalent parallel resistance R and the capacity C fall off to constant and very low values above 10,000 cycles, where the impedance (Z) of the large cell capacity is negligible (The impedance Z is derived from R and C according to the formula in the text.) On the other hand the same cell when killed (s) and its cellulose wall (W) cleaned and inflated with air, show very low and high resistances respectively. W and s ordinates in ohms only, they have no appreciable capacity at any frequency.

The impedance, which can be readily derived from this circuit by the formula *

$$Z = \frac{R}{\sqrt{1 + (2\pi fCR)^2}}$$

falls off rapidly with the increase of frequency, from zero (D.C.) up to some 10,000 cycles above which it is nearly constant. This is shown in Fig. 1.

The constant minimum value reached above 10,000 cycles is, of course, essentially that of the cell interior, here largely the sap of the vacuole. This is shown by readings taken with the same cell when killed, these have the same low value at all frequencies (now without capacitative components), as shown on Fig 1. Finally, if the sap be removed and the cell wall (wet with sea water) blown up with air, the value for the shunt resistance around the protoplasm is found. This is very close to the D C value of the living cell but remains unchanged at all frequencies, again without capacitative effects (Fig 1). The sap value and the cell wall value thus set the lower and the upper resistance limits, for the highest and lowest frequencies respectively. These are both purely ohmic resistances, without capacity, showing that the latter is due to the living protoplasm.

The low, but nevertheless appreciable value of the internal or vacuolar resistance suggests at once that the simple balancing circuit A used for Fig 1 is an inadequate representation of the cell, since it omits any resistance (r) in series with the capacity C . This has, of course, long been appreciated, and some type of series-parallel circuit which includes such a second resistance has been often postulated⁸ as the simplest equivalent of the cell. Such a one is shown on Fig 2, and may be called Circuit B. (A single capacity C is used, to represent the two series capacities of the cell due to the protoplasm at each end.) Both theoretically and experimentally, Circuit B gives the general type of impedance curve shown in Fig 1, when balanced against Circuit A. It may therefore be taken as a first approximation to the cell, and may be used instead of Circuit A to balance the cell directly in the bridge. This was accordingly done next.

In adjusting this circuit, W is set equal to the D C resistance, (that of the leakage around and through the cell). It is usually between 1000 and 5000 ohms with intact *Valonia* cells, depending on their dimensions. C and r are then adjusted to balance the bridge at any

⁸ Cole, K S, *J Gen Physiol*, 1928-29, **12**, 37, 1934-35, **18**, 877. Cole, K S, and Cole, R H, *J Gen Physiol*, 1935-36, **19**, 609, 625. Fricke, H, *J Gen Physiol*, 1923-24, **6**, 375, 1925-26, **9**, 137. Gildemeister, M, in Bethe, A, *Handbuch der normalen und pathologischen Physiologie*, 1928, **8**, part 2, 657. McClen-don, J F, *J Biol Chem*, 1925, **63**, 14, 67, 7, 1926, **68**, 653, 69, 733. Philippson, M, *Compt rend Soc biol*, 1920, **83**, 1399, *Bull cl Sc Acad Roy Belgique*, 1921, **7**, 387.

given frequency. The results of a typical run are shown in Fig 2, plotted against the frequency.

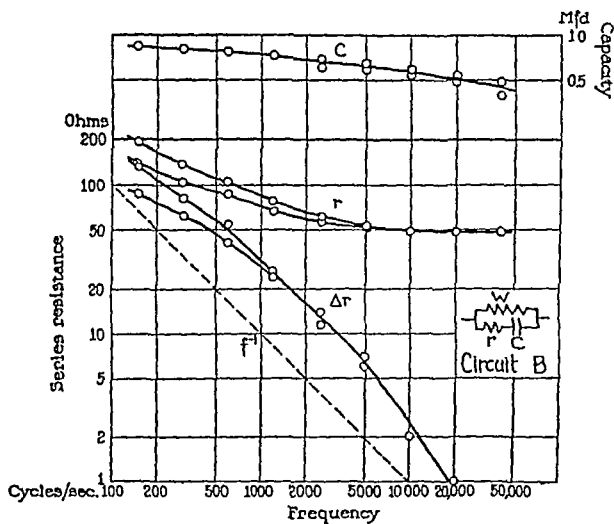


FIG 2 Characteristics of a *Valoma* cell bridging an air gap between two agar contacts when balanced against the series parallel Circuit B as shown. W is set at the D.C. value as representing the shunt resistance around the cell, and r and C are adjusted to give balance. Both still change with frequency, although much less than in Fig 1. (The diverging curves at low frequencies represent 2 runs, up and down, the frequency scale). Since r approaches a constant value above 10 000 cycles this constant value is assumed to be the ohmic resistance of the cell interior (sap) and is subtracted from the total value of r to give Δr as plotted. This has a slope approximating that of f^{-1} (ordinates for f^{-1} omitted) included for comparison but is markedly curved downward. Logarithmic scale.

It is obvious that a complete balance is not yet obtained, for both C and r values still change with frequency. If the minimum r value at high frequency (representing the ohmic resistance, S , of the sap, plus a small external resistance in series) is subtracted from the total

r setting, a value Δr is obtained, representing the purely protoplasmic resistance effects (This is the "equivalent series resistance" of polarization studies) This is inserted in Fig 2 It is seen that both capacity C , and Δr change approximately with the logarithm of the frequency If the plots were strictly linear, then if C_1 and C_2 are the capacities, and Δr_1 and Δr_2 the series resistances at two frequencies f_1 and f_2 , the relation would be

$$C_2 = C_1(f_2 - f_1)^{-x}$$

and

$$\Delta r_2 = \Delta r_1(f_2 - f_1)^{-y}$$

with x having a value between 0.1 and 0.3, and y nearly 1.0 In other words Δr varies nearly inversely with frequency, while C is an inverse (fractional) power function of the frequency However, both exponents change over the frequency range studied, giving curves rather than straight lines

Comparison with Electrode Polarization

Now this relation to frequency is almost exactly that which is well known to obtain with polarizable electrodes⁵ An example taken with the same bridge, using small platinum electrodes dipping in KCl solution (0.5 M), and shunted by 1000 ohms resistance (to duplicate the cell wall shunt) is shown in Fig 3 Even the slopes are nearly the same as with the *Valonia* cell The resemblance is so striking as to suggest that the *Valonia* capacity is really of the polarization type

Before this can be considered proved, however, another complication remains to be discussed

Distributed Capacity

By this is meant the capacity exhibited by the protoplasm not directly adjacent to the end contacts (which is perpendicular to the main axis of current flow), but by the protoplasm lining the cell wall between the contacts and hence parallel to the axis It might be thought at first glance that this would play no rôle, because it separates two uniform conductors, the sap and cellulose wall, any two corresponding points of which are equipotential (Thus the cell might

be imagined a miniature Wheatstone bridge with two slide wires one of large and one of small cross section but both uniform, a detector connecting any two points equidistant from the end would draw no current because the ratios of potential drop along the wires corre

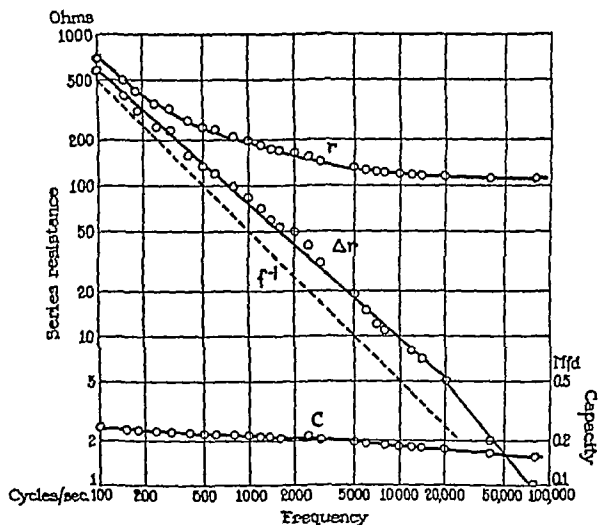


FIG 3 Characteristics of a pair of small platinum electrodes dipping in 0.5 M KCl, and shunted by a resistance W (representing the cell wall) balanced against Circuit B. Δr derived by subtracting the value of r reached above 100,000 cycles from that at a given lower frequency. The changes of C , r , and Δr with frequency resemble those of the *Valonia* cell in Fig 2, but the slope of Δr more nearly approximates that of f^{-1} as shown. The balancing Circuit B is shown on Fig 2.

spend) This would be true if there were no perpendicular capacities at the contact ends. But the presence of these in one of the current branches (protoplasm sap) and its absence in the other (wall), introduces a non uniformity in the former, so that only at the center of the cell, half way between the contacts (assuming equal capacities

at each end), would this branch be equipotential with the outer or wall branch (Only here would the cell as a Wheatstone bridge be in balance) At all other points toward either contact, there would be an unbalance, a difference of potential, and a tendency for current flow between wall and sap If this flow were merely across a resistance it would not be so troublesome, but it is across protoplasm, hence across a capacity, which would increase the total apparent capacity of the system as measured Even if it only did this, the error would not be serious, since we are not so much interested in absolute values per cm^2 as in the changes with frequency But unfortunately the case is not so simple For the very reasons which make the distributed capacity effective, a change of frequency will change the magnitude of its effects This results from the decreased impedance of a capacity with frequency $\left(Z = \frac{1}{2\pi f c} \right)$, so that the higher the frequency, the less the impedance of the protoplasm at the ends of the sap-protoplasm circuit, and the less the non-uniformity introduced by it Consequently at higher frequencies, less current will tend to flow across the distributed capacity, until above 10,000 cycles where the impedance of the contacts becomes negligible, practically no current will flow across the distributed capacity Its contribution to the total capacity will thus decrease and there will be a decrease of measured capacity with frequency

That such a circuit can actually give rise to these effects is shown by a simplified model This consists of Circuit B, with the addition of a second fixed mica condenser connecting the mid-points of W and r This condenser may be regarded as a first approximation to a capacity uniformly distributed between W and r , as in the nominal "T-network" used in telephone and other cable problems⁹ This results in Circuit C, as shown in Fig 4 When this circuit, embodying the capacity and resistance values given in the legend, is balanced against the simple series-parallel Circuit B, it is seen that C and r of the latter circuit now change with frequency, as plotted in Fig 4 (It should be emphasized that the mica condensers employed were of

⁹ Kennelly, A E, *Electric lines and nets* Their theory and electrical behavior, New York, McGraw-Hill Co, 2nd edition, 1928

good quality, and showed no such changes with frequency unless connected in this fashion)

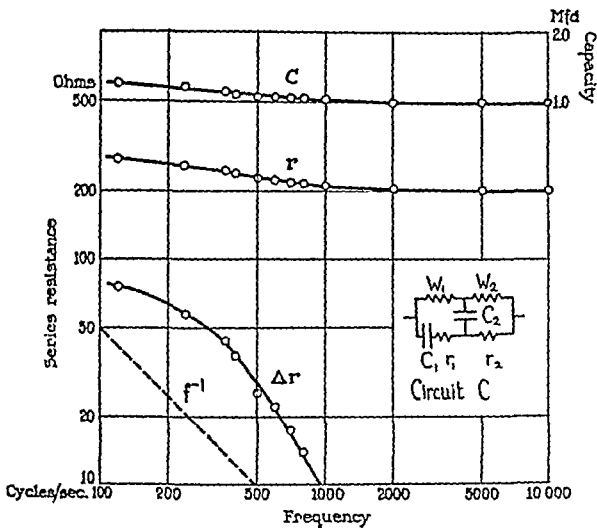


FIG. 4 Artificial Circuit C (as shown in the figure) containing a T network approximating the distributed capacity along half the cell, balanced against Circuit B. The values of C_1 and C_2 in Circuit C were each 0.98 mfd (high grade mica condensers), R_1 and R_2 each 1000 ohms, r_1 and r_2 each 100 ohms. r derived as previously by subtracting the constant value of r , here reached above 2000 cycles, from the value at a given frequency. The change of C , r , and Δr with frequency somewhat resembles those obtained with *Valonia* cells (Fig. 2) and those with polarizing electrodes (Fig. 3) although the capacities in model Circuit C are purely static showing no such changes unless arranged in this approximation to a distributed capacity. The marked curvature of Δr to the slope f^{-1} should be noted (ordinates for f^{-1} are omitted). The balancing Circuit B is shown on Fig. 2. Logarithmic scale.

While there are divergences from cell behavior, such as the flattening out of the capacity curve at higher frequencies, and the exaggerated

Δr plot, extremely curved to the slope f^{-1} , the trends are nevertheless sufficiently alike to raise serious question as to whether some at least of the apparent polarization effects may not be ascribed to a distributed capacity of static type

It therefore became extremely desirable to determine the magnitude of this distribution effect in *Valonia*. Several methods suggested themselves, including

- 1 Direct comparison of the cell with a model such as Circuit C in the balance arm. This was not attempted because of a lack of sufficient variable elements to make up the circuit, in any case it would only be approximate because Circuit C is but a first approximation to true distribution, which involves hyperbolic functions⁹

- 2 Calculation of the effect from the available dimensions of the cell, and its resistance and capacity constants. This is rendered difficult because of irregularities of the cells, which are seldom strictly cylindrical between the contacts

- 3 Reduction or avoidance of distribution by proper experimental arrangement. This is the most direct solution, and was accomplished in two ways: by greatly shortening, or abolishing the air-gap between the contact regions, thereby reducing the length of the cell over which distributed capacity could prevail, or by using impaled cells where the current must all flow radially across the protoplasm, with no opportunity for distributed capacity

Reduction of the Air-Gap

This experiment may be done in one of several ways: (a) The cells may be inserted farther into the holes of the agar contacts, so that the latter are separated by an air-gap of only 1 or 2 mm. instead of the customary 1 cm. (b) They may be placed in a circular hole which just accommodates them, in a thin septum of wax or other non-conducting material separating two chambers of sea water. (c) They may be floated loosely in a tube of sea water somewhat larger than their greatest diameter. Each of these methods gives progressively larger contacts with less distributed capacity between them. On the other hand the decrease of shunting resistance around the cell diminishes the change of impedance with frequency because the original direct

current value is lower, this blunts the sensitivity of measurements. However, for what it may be worth, as an indication of the change

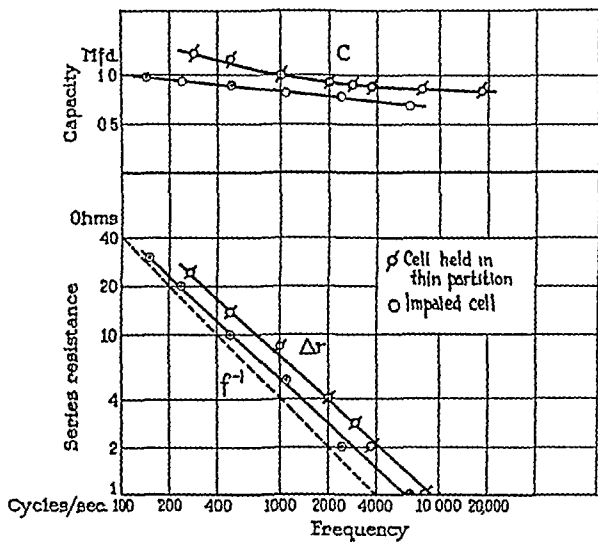


FIG 5 Characteristics of a *Valonia* cell arranged to reduce the effects of distributed capacity, either by abolishing the air gap (cell instead held in an aperture of a thin wax partition) or by impaling the cell on a capillary. r values are omitted to avoid confusing the graph, Δr being derived from these as before. Both C and Δr still change with frequency although the slope of C is less, and the curvature of Δr with respect to f^{-1} is lost (ordinates for f^{-1} are omitted). The results are consistent with the presence of some polarization capacity in the cells, showing that distributed capacity is not entirely responsible for the effects in Fig 2. The balancing Circuit B is shown on Fig 2.

produced by decreasing the distributed capacity, the readings of a B circuit, balanced against a cell so arranged (here held in a partition between two compartments) are given in Fig 5.

Measurement of Impaled Cells

This method should give no distributed capacity because the current flow is entirely radial across the protoplasm. It has special difficulties, however, which diminish its apparent advantages. Chiefly there is the risk of injury, or alteration of polarization properties as the result of inserting the necessary fine glass capillary. D C records, taken by methods previously described,⁴ were therefore essential in order to ascertain the presence of "regular" polarization. Usually cells which had spontaneously attained the regular state were used, and are the only ones reported here. In a few cases comparative measurements were taken with cells made regular by exposure to slightly acidified sea water, or "conditioned" by the continuous flow of direct current inward across the protoplasm in the manner noted earlier.⁴ In the latter case, the proper conditioning current was produced by inserting sufficient E M F in one arm of the bridge in series with the cell, this avoided the use of troublesome coils and condensers to separate A C and D C components in the bridge input and output.

Another difficulty is the necessarily high series resistance introduced by the fine glass capillary used for impalement.¹⁰ With the shortest and broadest of these which could be safely inserted, the resistance was seldom less than 5,000 ohms (when filled with *Valonia* sap). This high value naturally tends to obscure the changes in the protoplasmic series resistance Δr , which usually does not change more than a few hundred ohms with frequency over the range used. Careful attention to temperature control was therefore necessary.

The balancing arm of the bridge was the series-parallel circuit (B) previously used, with the addition of an external series resistance (Y) representing the capillary. Y is determined, not from the capillary value before impalement, but, lest this alter somewhat, from the high frequency (20,000 cycle) readings with the impaled cell at the time of measurement, the impedance of the protoplasm being negligible at this frequency. W is set as the difference between Y and the total direct current resistance of the system with an impaled cell.

¹⁰ It might be thought that the introduction of true micro-electrodes such as fine metal wires or metallized needles of glass might overcome this difficulty. They were tried, but introduced more difficulty than they solved, by their own capacity effects, due to polarization at their small surfaces.

Since W now represents only the leakage resistance across the protoplasm itself, it runs much higher than with intact cells, (where it was largely that of the shunting cell wall) and values of 10,000 to 20,000 ohms are not exceptional

With W and I fixed, C and r are adjusted as before to give a balance at any given frequency. Resulting values are shown in Fig 5. It is seen that there are still changes with frequency, although the capacity is more nearly constant than with intact cells. The slope of Δr is about the same as before, but is now nearly straight and parallel to f^{-1} , showing that distributed capacity was apparently responsible for its curvature. It may therefore be concluded that a frequency dependence of capacity and of its associated series resistance really occurs in *Valonia* cells, and is not entirely due to distributed capacity. The rôle of the latter should not be neglected, however, and should be considered in other cases where contact is made at the ends of long cylindrical cells (such as *Nitella* or nerve)

DISCUSSION

The residual change of capacity and resistance with frequency indicates that some sort of polarization phenomenon occurs in the protoplasm. Beyond this statement it is unwise to go for the present, since neither polarization theory for electrodes, nor experimental evidence for phase boundaries of lipoids, etc. is advanced enough to help the interpretation. The slope of capacity against frequency is somewhat less than has been found with various electrodes,⁶ but even these are variable, and thorough study is much needed. A change of capacity with frequency is even known to occur in poor static condensers (e.g. of paraffined paper),¹¹ but this may be due to polarization of ions contained in the dielectric.

The results suggest the following tentative situation: a partly static capacity, constant with frequency, in parallel with some type of polarizing system having a capacity and series resistance variable with frequency. The relative magnitudes of these would determine the resultant measured slope with frequency, these may be somewhat variable from cell to cell, just as they evidently are from organism to organism (compare the nearly constant static capacity of erythro

¹¹ Grover, F. W., *Bureau Standards Bull.*, 1911 7, 495

cytes and sea urchin eggs with the frequency dependent values in muscle⁸) Such properties could result from a cell surface (*e g* lipid) acting partly as the thin dielectric of a condenser, but having likewise a differential, ionic permeability The latter could be due either to scattered ion-permeable regions ("pores") occupying part of the surface, or to a low but general solubility of ions in the surface as a whole (In a monomolecular lipid film these might amount to the same thing) This is the picture known as the "lipoid-sieve" hypothesis It is consistent with much other evidence showing the cells to be most readily permeable to non-ionized, lipid-soluble substances, while still displaying electrical properties such as (low but appreciable) conductance, polarization, and potential differences (The pore theory, however, is not consistent with the dilute solution being positive with KCl and negative with NaCl with *Valonia* ¹²) The question arises, which ions are responsible for the polarization phenomena? If this could be determined, it might be possible for example, to change the protoplasm from an irreversible electrode to a reversible one by changing external solutions, with resulting effect upon the frequency dependence of capacity ⁵

The most promising suggestions come from effects upon potential difference For example, differential ionic mobilities have been postulated in *Valonia*¹¹ to account for the effects of various solutions on the *p D*, K ion being assigned a high and Na ion a low mobility in relation to Cl Measurements made, however, on the *A C* capacity of cells exposed to KCl, as well as *D C* records,⁴ indicate that there is very little if any difference in the shape of the polarization curves obtained in the presence of KCl and of ordinary sea water, at least for considerable time The possibility therefore, that the polarizations are largely due to organic ions produced in the metabolism of the cell itself, should not be overlooked In this case external changes would not have much effect

Grateful acknowledgment is made to the Carnegie Institution of Washington for opportunities to study at its Dry Tortugas Laboratory

SUMMARY

Alternating current measurements of the effective capacity and

¹² Damon, E B, *J Gen Physiol*, 1932-33, 16, 375, and Osterhout, W J V, *J Gen Physiol*, 1929-30, 13, 445

resistance of *Valonia* cells were undertaken to determine whether a static or polarization capacity was responsible for the large slow time curves of counter E M F produced by the flow of direct current (For this purpose it was necessary that the cells be in the regular state) With external contacts at the ends of cells, a large fall of impedance occurs over the frequency range from zero to 20,000 cycles, above which the impedance is low and essentially that of the cell interior

As a first approximation to the cell circuit, a simple series parallel circuit was employed in the bridge balance, with a resistance setting to represent the cell wall (and protoplasmic leakage), shunting the protoplasmic capacity in series with a resistance (sap plus polarization resistance) Both the capacity and its associated series resistance fall off regularly with frequency, giving curved lines on logarithmic plots against frequency, the slope of the resistance plot being steeper, and approaching that of f^{-1} , although curved to it These parallel roughly the behavior of a polarizing electrode, which is also shown

Before concluding that the cell's capacity is therefore due to polarization, a further complication of the circuit was considered This was the effect of the protoplasmic capacity distributed along the wall between the contacts Both logically and experimentally it was shown that a distributed capacity, as represented by its approximate T network of resistances and capacities invariant with frequency, could give rise to changes of capacity and series resistance with frequency which simulate to some extent the cellular phenomena

Distributed capacity was therefore reduced in the cells by using shorter air gaps between the contacts, or abolished by measuring impaled cells, in which the current flow across the protoplasm was entirely radial These measurements showed a smaller, but still significant change of capacity and of equivalent resistance (in series with it) with frequency, somewhat less than with electrodes, and probably representing the true protoplasmic behavior It is concluded that the cells display a certain degree of polarization capacity, possibly in parallel with a static capacity invariant with frequency This might result from an insulating (e.g. lipid) cell surface, having a residual differential permeability to ions This structure is consistent with other evidence showing the cells to be chiefly permeable to non ionized, lipid soluble materials, but still displaying electrical effects (conductance, potential difference, polarization) ascribable to ionic mobility

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STIMULATION OF FUNDULUS BY OXALIC AND MALONIC ACIDS AND BREATHING RHYTHM AS FUNCTIONS OF TEMPERATURE*

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(Accepted for publication August 24, 1935)

CORRECTIONS

In Vol 19, No 4, March 20, 1936 page 603, in the eleventh line from the bottom of the page for ' $z_i = 2, z_{ii} = 1$ ', read ' $z_i = 1, z_{ii} = 2$ '

On the same page in the fourteenth line from the bottom of the page for ' $_{jj}(\text{HPO}_4^-)$ ' read ' $_{jj}(\text{HPO}_4 -)$ '

On page 685, at the end of the fourth line of the legend for Fig 4, the sentence should begin " Δr derived" instead of ' r derived

On page 690 in the paragraph beginning in the middle of the page, in the second sentence the reference should be to footnote 12 instead of to footnote 11

might vary in a measurable manner with temperature It was hoped

* This report is a portion of the thesis submitted in partial fulfillment of the requirements for the degree of Ph D at Rutgers University, 1935 I wish to express my sincere thanks to Dr William H Cole and Dr J B Allison for their great helpfulness during this research

† Part of this work was carried on at the Mount Desert Island Biological Laboratory

that the relations of temperature to the quantitative response to chemical stimulation might be used to analyze the connection between properties of the stimulant and the amplitude of reaction as measured by rate of response. Several questions of theoretical significance regarding chemical stimulation might be answered by such an attack. In certain studies on chemical stimulation, factors related to the surface tension of the stimulating agent have been found to be of importance (Cole, 1931-32). A temperature study might reveal whether these are controlling agents in chemical stimulation. It has been shown elsewhere (Sizer, 1934, also Allison and Cole, 1933-34) that a parabolic relationship exists between rate of response and (H^+) for stimulation of *Fundulus*. Adsorption also varies parabolically with concentration. Is the adsorption of acid molecules at the receptor interface a necessary preliminary to stimulation, and if so is this the controlling process determining rate of response? Cole and Allison have stated (1932-33) that since the coefficient of variability (*i.e.* probable error expressed as per cent of the mean) does not vary with reaction time, the mechanism of the reaction to a given acid is the same regardless of the concentration, within the experimental limits. Experiments designed to furnish thermal increments for stimulation by different concentrations of the same acid should show definitely whether the mechanism of reaction changes with concentration. Oxalic, malonic, and succinic acids give different constants in the parabolic equation relating rate of response to (H^+) . Does this mean that the fundamental architecture of the stimulation mechanism is the same for the three acids, and that the chemical reaction controlling the rate of the reaction processes is different in the three cases? Stimulation by the dicarboxylic acids in fresh water has been found to be different from stimulation in salt water, although in both cases parabolic relationships were found. Is this evidence of a difference in the chemical processes controlling rate of stimulation in the two environments? An answer to these questions might be indicated by an investigation of the relationship between acid stimulation and temperature.

Method

The experimental procedure was the same as that for stimulation of *Fundulus* by the dicarboxylic acids and their derivatives (Sizer, 1933, 1935). The fish

was placed in a small celluloid reaction chamber through which solutions were passed at the rate of 100 ± 5 cc per minute. To make a test the salt or fresh water was turned off, and the acid solution turned on at the same rate of flow and at the same temperature. The response was measured with a stop watch. The temperature was held constant to $\pm 0.1^\circ\text{C}$. Ice was used as the cooling agent for tests made in salt water below 12°C . When ice was used the temperature sometimes varied by as much as $\pm 0.2^\circ\text{C}$. Ample time was allowed the fish for adaptation at a given temperature. This time varied from a few minutes at room temperature to several hours at low temperatures. The aquaria containing the individual fish were immersed in the water bath so that the fish might be kept at the desired temperature for an ample period. When the fish was removed to the reaction chamber for stimulation it was already adapted to the experimental temperature. Each fish was stimulated at 2 minute intervals at all temperatures. This recovery time was ample since there was no progressive change in reaction time due to adaptation to the stimulus. For the tests made in fresh water ten reactions were taken on each of six fish at a given temperature while for salt water work twenty reactions were taken on each of three fish. In both environments sixty readings were taken for each temperature. Above about 15°C the fish were quite active and moved about unless held in position with a wire screen. Below this temperature the fish were very quiescent, scarcely moving even a fin over long periods of time. At high temperatures cessation or change in rate of opercular movements was the criterion of response, but at low temperatures the first visible unusual movement, whether opercular or not, was considered the response to stimulation. To minimize the personal element as much as possible the range of temperature was studied at 2° intervals, the range of temperature was then covered again, this time response to stimulation being determined at the intervening temperatures. The results were not analyzed until the end of the experiment so that the observer would not be prejudiced in taking readings. Fish occasionally died over a period of a few weeks and were replaced by new ones. Since the variation in individual reaction times is not great this procedure did not noticeably affect the average reaction time. The pH of the solutions both in fresh and salt water was measured daily by the quinhydrone electrode.

It was necessary to take special precautions to make sure that the stimulating solution entered the reaction chamber at exactly the same temperature as the salt or fresh water which it displaced. Otherwise a distinct response of the fish was noticed but here the stimulating agent was the temperature change, not the acid solution. This response to temperature change suggests a new series of experiments where the stimulating agent is salt or fresh water adjusted to various temperatures and passed in to the reaction chamber which is held at a constant temperature. A modification of this experiment would be to vary the temperature of the reaction chamber as well.

Experiments in Fresh Water

Acid stimulation of *Fundulus* as related to temperature was studied by using as the stimulating agent two widely different concentrations of oxalic acid. 0.002N oxalic has a pH of 3.14 and gives a reaction time of the fish of 5.5 seconds at 18°C. 0.0008N oxalic has a pH of 3.82 and gives a reaction time of 11.8 seconds at 18°C. Stimulation by these acids was tested at temperatures ranging from 1–30°C. The reaction time was corrected as before (Sizer, 1934, also Allison and Cole, 1933–34) by a subtraction of 4 seconds, and then log rate of response was plotted against the reciprocal of the absolute temperature. Each point represents the average of sixty readings, ten taken on each of six fish. This averaging is justified due to the small variability of reaction time among the several fish. An analysis of the graphs made with data from individual fish, as well as the graph made from the mass plot of the data from individual fish showed good agreement with the analysis made on the basis of the averages.

Two straight lines intersecting at the critical temperature, 6.5°C, may be drawn through the points plotted for 0.002N oxalic (Fig. 1). The line drawn for the lower temperatures has a slope represented by the μ value, 33,000, the line for the higher temperatures gives a $\mu = 15,800$. At 24°C and above the reaction time reaches a minimum and constant value. Below 6.5°C there is an increased scatter of the points indicative of the fact that a different chemical reaction is in control. An increased scatter of points is also noticed at temperatures above 20°C.

A similar relationship is obtained for stimulation by 0.0008N oxalic as related to temperature (Fig. 1). The curves are displaced along the y axis, but the lines drawn through the experimental points are parallel to those for 0.002N oxalic. However, these lines intersect at 10.5°, instead of 6.5°. This indicates that the shift in the controlling chemical reactions which regulate the rate of response occurs at a significantly higher temperature for 0.0008N oxalic as compared with 0.002N oxalic. A possible explanation for this shift in critical temperature with change in concentration is suggested. If a definite energy level must be reached in order to bring about a change in the slowest or controlling reaction regulating rate of response, then this

certain energy level would be reached at a lower temperature for 0.002N oxalic than for 0.0008N oxalic. Thus 0.002N oxalic would have the lower critical temperature. The reaction time for 0.0008N oxalic does not reach a minimum value at 24°C, but continues to decrease

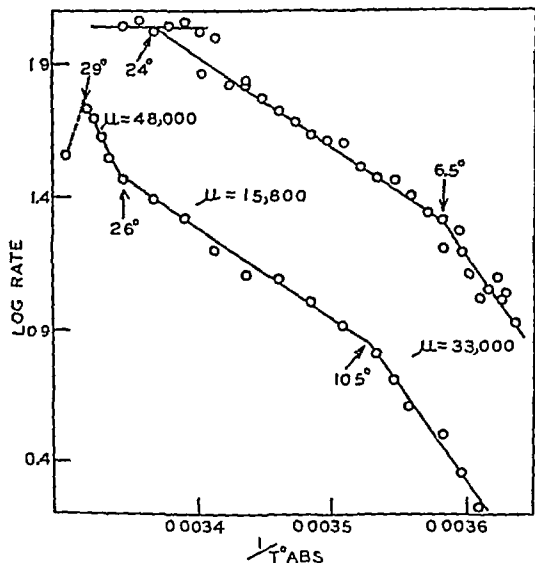


FIG 1 Log rate of response ($\log \frac{100}{R(T-4)}$) of *Fundulus* in fresh water to stimulation by 0.002N oxalic (upper curve) and 0.0008N oxalic (lower curve) plotted against the reciprocal of the absolute temperature. The curves are parallel except at high temperatures but the critical temperatures are different.

with increase in temperature. At 26°C a new critical temperature is encountered. A line drawn through the points between 26°C and 29°C gives a $\mu = 48,000$. Beyond 29°C toxic effects set in and the reaction time becomes longer again. A similar temperature char-

acteristic for 0.002N oxalic would be expected above 26°C were it not for the fact that the reaction time has already reached its minimum value at 24°C. Since the thermal increments are the same, it may be stated that the mechanism of reaction does not change when the concentration of oxalic is changed from 0.002 to 0.0008N. Differences existing between stimulation by these two concentrations of oxalic as a function of temperature may be explained on theoretical grounds.

Experiments in Salt Water

Stimulation was measured in salt water at temperatures ranging from 0 to 30°C. At each temperature sixty reactions were taken, twenty on each of three fish and the results were averaged. An analysis of the data for individual fish showed good agreement with the analysis made on the averaged data. Thermal increments determined for the separate fish varied somewhat, but were not significantly different from those determined for the average values.

(a) 0.002N Oxalic — 0.002N oxalic in salt water has a pH of 5.40 and gives a reaction time of 8.7 seconds at 18°C. Stimulation by this acid as a function of temperature is characterized by three different thermal increments (Fig. 2, lower curve, upper curve refers to variability which will be discussed in another section). Over the temperature range of 1–3.3°C $\mu = 56,500$, from 3.3–15°C $\mu = 19,400$, and from 15–29°C $\mu = 24,100$. At 15°C there is not only a change in increment but a change in rate of response as well. It will be noticed that the points for 20, 22, and 24°C, do not lie along the line as drawn. However, a line drawn through these three points would be parallel to the curve as drawn. It happened that these three points were determined consecutively, the reaction time of the fish had decreased but there was no change in temperature characteristic. This is an example of what Crozier and Stier (1926–27) have called a change in frequency without change in increment. It may be noticed that the μ value is higher for the range from 15–29°C than it is from 3.3–15°C. This is the reverse of the usual situation for the temperature characteristics at high and low temperatures. Both thermal increments and critical temperatures are different for stimulation by 0.002N oxalic in salt water and 0.002N and 0.0008N oxalic in fresh water. While the mechanisms for stimulation are similar in both environments, as evi-

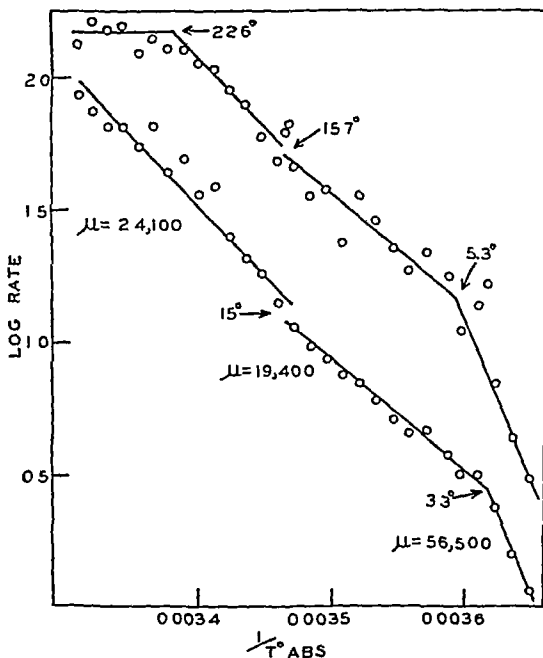


FIG 2 Upper curve Ten times the reciprocal of the probable error of the response of *Fundulus* in salt water to stimulation by 0.002N oxalic plotted against the reciprocal of the absolute temperature

Lower curve Log rate of response $\left(\log \frac{100}{R(T-4)}\right)$ to the same acid plotted against $\frac{1}{T}$. The two curves are parallel but the critical temperatures differ

denced by the fact that parabolic equations for stimulation are obtained in both cases, the chemical reactions governing the rate of response are different in the two environments. This difference in

stimulation in salt and fresh water is doubtless correlated with the difference in ionic constitution of the two environments (Allison and Cole, 1933-34) The complex ionic equilibrium at the receptor interface would be altered in quite a different manner by the addition of oxalic to a salt water environment, than it would by adding oxalic to fresh water. Addition of 0.002N oxalic to salt water causes a small amount of calcium oxalate to be precipitated, CO_2 is liberated, but since salt water is highly buffered the (H^+) does not change very greatly The same acid concentration in fresh water brings about a much greater percentage increase in the ionic concentration of the environment, a much greater increase in (H^+) , and a greater acid anion concentration, than it would in salt water

(b) 0.002 and 0.004N Malonic —Stimulation by malonic acid in salt water as related to temperature was tested by using 0.004 and 0.002N solutions 0.004N malonic has a pH of 4.11 and gives a reaction time at 18° of 6.0 seconds 0.002N malonic has a pH of 5.64 and gives a reaction time of 6.9 seconds at 18°C It would have been better if a solution more dilute than 0.002N had been used, but practically it is very difficult to measure response to such a weak solution An analysis of the data shows similar results for the two different concentrations of malonic acid (See Fig. 3) In both cases the μ values are 65,000, for the lower range, and 20,600 for the upper range of temperatures For 0.004N malonic critical temperatures exist at 6.3°C and 23°C The reaction time is constant above 23°C Critical temperatures exist at 6.4°C , and at 25°C for 0.002N malonic The reaction time becomes constant above 25°C For oxalic acid in fresh water and malonic acid in salt water the statement may be made that over the concentration range studied the mechanism of reaction is independent of the concentration for a given acid and a given environment However, the master chemical reaction, the slowest process in the catenary series of events controlling rate of response, is different for stimulation by oxalic acid in the two different environments A comparison of the temperature characteristics and critical temperatures for stimulation by oxalic and malonic in salt water shows a distinct difference This can only indicate that the mechanism of reaction is different for these two members of the dicarboxylic acid series However, since both acids yield parabolic equations for stimulation it must be assumed

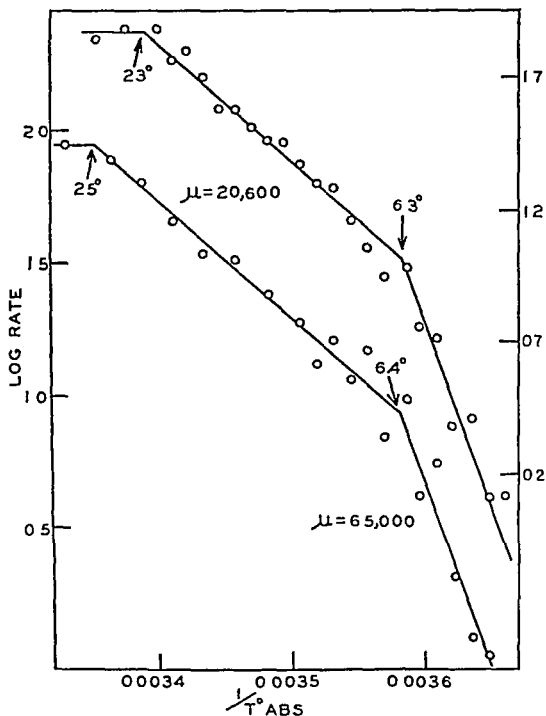


FIG 3 Log rate of response ($\log \frac{100}{R T - 4}$) of *Fundulus* plotted against reciprocal of absolute temperature. Upper curve: Stimulation in salt water by 0.004N malonic. Lower curve: Stimulation in salt water by 0.002N malonic. The two curves are parallel but the critical temperatures differ.

that the fundamental stimulation system is the same for the two acids, and that the difference between the two systems is related to the nature of the chemical reactions which determine the rate of response to acid stimulation.

The critical temperatures for stimulation by 0.004 and 0.002N malonic are practically the same. Moreover their stimulating efficiencies as measured by reaction time are not widely different. If there were a greater difference in the (H^+) of the two concentrations of malonic, a greater difference in critical temperatures might be expected corresponding with the situation for stimulation by two different concentrations of oxalic in fresh water.

DISCUSSION

It was hoped that a study of stimulation as a function of temperature would indicate the nature of the events occurring between the orientation of the acid molecules at the receptor-environment interface and the response of the fish expressed as a change in opercular rate. It has already been shown that stimulation by the dicarboxylic acids and their derivatives is not directly correlated with the surface tension. The fact that it is not a primary factor in stimulation is shown as well by the thermal increments for stimulation by oxalic and malonic acids, they are much higher than those typical of surface tension phenomena. Diffusion of the acid molecules to or through the receptor surface may also be ruled out for the same reason as a primary factor determining rate of response. The adsorption of the acid molecule as a possible preliminary to stimulation was suggested because both rate of response and degree of adsorption are parabolic functions of concentration. While adsorption may be involved, it is not a controlling factor determining rate of response. The μ values are all above 15,000 and clearly show that the factors controlling the rate of response to acid stimulation are not physical but rather chemical in nature.

Several different temperature characteristics have been obtained for stimulation by oxalic in fresh and salt water, and for malonic in salt water. This indicates that stimulation does not depend upon a single sort of process, but rather upon a series of interrelated chemical reactions, each with its own velocity constant. Under varying conditions different chemical reactions may become the slowest or controlling process which determines the rate of response. It might be expected that new μ values would be revealed by testing stimulation by other acids in fresh and salt water as a function of temperature. Higher members of the dicarboxylic acid series give the same parabolic

equation for stimulation (Sizer, 1934) It would be of great interest to know whether or not stimulation by these acids yields identical relationships to temperature

Little can be said about the actual values of the thermal increments for stimulation by oxalic and malonic acids (see Table I) There are two cases where the μ values are greater for a high range of temperature than they are for a lower range This is the reverse of the customary situation The actual temperature characteristics which are found for stimulation by oxalic and malonic acid are not uncommon and are the same as those found for certain other biological processes, indicating similar chemical systems for many biological reactions It is interesting to observe that all the μ values under 40,000 which are

TABLE I
Temperature Characteristics for Stimulation of Fundulus

μ Value	Acid	Environment	Temperature range
15 800	0 002N 0 0008N oxalic	Fresh water	6 5-24 10 5-26
19 400	0 002N oxalic	Salt water	3 3-15°
20 600	0 004N 0 002N malonic	Salt water	6 3-23 , 6 4-25
24 100	0 002N oxalic	Salt water	15-29
33 000	0 002N 0 0008N oxalic	Fresh water	1-6 5 1-10 5
48 000	0 0008N oxalic	Fresh water	26-29
56 500	0 002N oxalic	Salt water	1-3 3
65 000	0 004N 0 002N malonic	Salt water	0-6 3 0-6 5

reported here for stimulation have also been observed for respiratory and oxidative phenomena (Crozier, 1924-25) Thermal increments above 45,000 such as those found for acid stimulation are very rare for completely reversible biological reactions

The critical temperatures for stimulation by oxalic and malonic are 3 3, 6 3, 6 4, 6 5, 10 5, 15, 24, 26, and 29°C These values are not uncommon for biological phenomena

It has been observed many times that the variability as measured by the relative scatter of plotted points frequently changes at critical temperatures Little has been done, however, to relate quantitatively such variability of a biological process to the temperature (Crozier, Stier, and Pincus, 1929) It has been shown (Stier, 1932-33, also

Navez, 1930) that the variability of the rate of a biological process bears a constant proportion to that rate. If this is true, then the variability, as measured by the probable error, of the rate of response to acid stimulation should be the same function of temperature as the rate of response itself. An analysis, then, of variability as related to temperature should be an excellent check upon the temperature characteristics and critical temperatures obtained by relating rate of response to temperature. It also follows that the coefficient of variability of the mean, or probable error expressed as per cent of the mean, should not vary with the reaction time, and when plotted against the reciprocal of the absolute temperature a straight line should be obtained having zero slope (Navez, 1930).

Probable errors were calculated for all the data obtained for fresh and salt water stimulation by oxalic and malonic acids¹. The actual magnitudes of the probable errors cannot be compared for the two environments, since six fish were used in fresh water tests and three in salt water tests. However, we are interested here only in relative values. Instead of plotting probable error against $1/T$, its reciprocal was used, so that the curves obtained might be more easily compared with those for rate of response. A comparison of the probable error curves (Figs. 2, 4, and 5) and those for rate of response (Figs. 1, 2, and 3) reveals an excellent agreement. The points for probable error of 0.002N oxalic in fresh water, however, are scattered, but tend to lie along the line which has the same slope as the curve for rate of response. The critical temperatures are within one or two degrees of those found for rate of response. It becomes apparent that variability of response time is not a haphazard affair but varies with temperature in a manner similar to rate of response itself.

The one striking difference between the series of curves for probable error and those for rate of response is that the variability becomes constant at lower temperatures than does the rate of response. This difference is not apparent for 0.0008N oxalic, for over the temperature range studied neither the rate of response nor probable error reach a constant and limiting value. For the other four acid concentrations the probable error reaches a constant value at a temperature five or

¹ Probable error = $\pm 0.8453 \frac{\sum(+V)}{n\sqrt{n-1}}$

six degrees lower on the average than does rate of response. A comparison of the rate of response and probable error curves for 0.002N oxalic stimulation in fresh water shows that above 20°C there is an

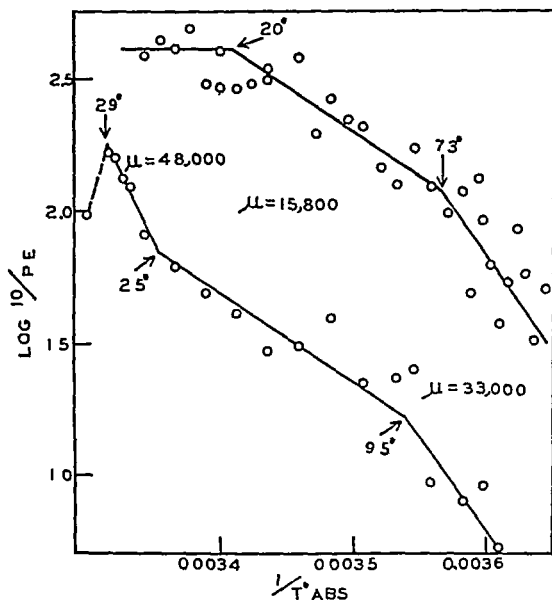


FIG. 4. Log ten times the reciprocal of the probable error of the response of *Fundulus* in fresh water to 0.002N oxalic (upper curve) and 0.0008N oxalic (lower curve) plotted against $\frac{1}{T^*}$. The curves are parallel except for high temperatures, and have the same slopes as the curves for stimulation by these acids. The critical temperatures are different, however.

increased scatter of points for rate of response. At this same temperature and above the probable error becomes a constant. The reaction time itself, however, does not become a constant until the temperature has reached 24°C.

Crozier has suggested that the variation in a biological process may be due to changes in the effective amounts of catalyst involved in the

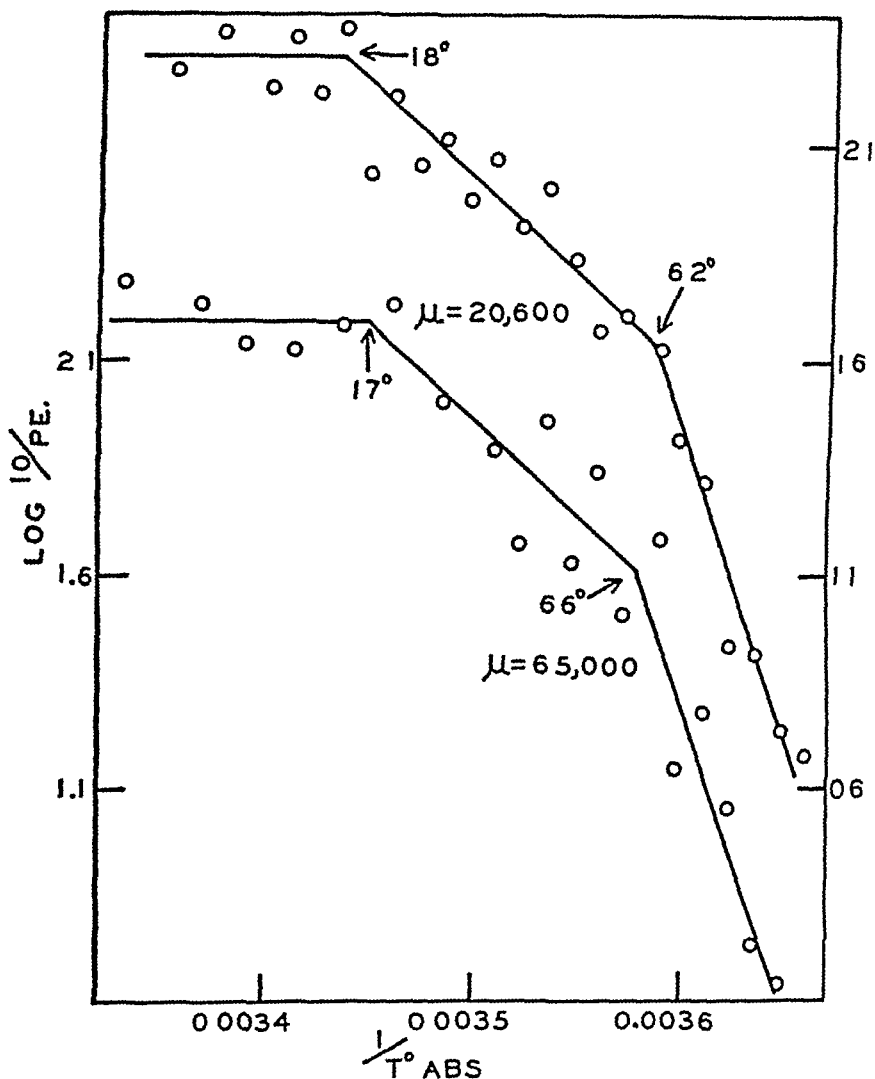


FIG 5 Log ten times the reciprocal of the probable error of the response of *Fundulus* in sea water to stimulation by 0.004N malonic (upper curve) and 0.002N malonic (lower curve). The curves are parallel and have the same μ values as those for rate of response to these acids. The critical temperatures are different, however.

chemical system determining the rate of that process. If the catalyst concentration concerned in the stimulation mechanism becomes con-

stant at high temperatures, then variability in rate of response will cease to vary as a function of temperature. The rate of response, however, is determined among other things by the magnitude of the initial disturbance in the chemical system as well as by the velocity

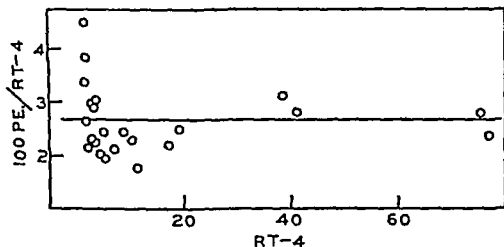


FIG 6 Per cent probable error of the corrected reaction time for stimulation of *Fundulus* in salt water by 0.004N malonic plotted against the corrected reaction time. The line drawn represents the average probable error of 2.71 per cent.

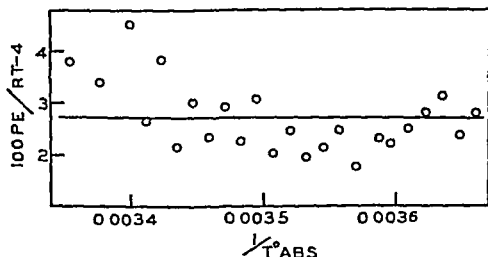


FIG 7 The same as Fig 6 plotted against $\frac{1}{T}$. The line drawn represents the average probable error of 2.71 per cent.

of subsequent reactions. This initial disturbance might continue to increase with temperature at temperatures even above those at which catalyst concentration and variability of response have become constant. Due to the physical limitations of the stimulation mechanism

the response time also reaches a limiting value at somewhat higher temperatures

Since the probable error curve follows that for rate of response the relationship

$$\frac{100 \text{ P E}}{R T - 4} = \text{Constant}$$

would be expected to hold. The truth of this statement is clearly shown by plotting coefficient of variation for stimulation by 0.004N malonic in salt water against reaction time (Fig. 6). This coefficient of variability appears to be roughly independent of the temperature as may be seen in Fig. 7. At temperatures above which the probable error has reached a constant and limiting value it ceases to be a constant per cent of the mean reaction time and increases in value. This is indicative of the fact that at these higher temperatures factors are affecting the rate of response of the fish which are not ordinarily related to the stimulation mechanism. This increased variability at high temperatures signifies a change in the mechanism of reaction to stimulation. The average value for the probable error expressed as per cent of the mean reaction time is 2.71 per cent for 0.004N malonic. A similar analysis would yield comparable results for the other acid solutions which have been studied.

The fact that the reciprocal of probable error is the same function of temperature as rate of response, and that the coefficient of variability does not vary either with reaction time or with temperature, should be interpreted as indicating that variation itself is not a variable changing independently with temperature. Variation in response as measured by probable error is a direct function of reaction time and is determined by the same catenary series of events which determine rate of response to stimulation.

Breathing Rhythm of Fundulus As Related to Temperature

In connection with the study of chemical stimulation as related to temperature it was thought necessary to make a simultaneous study of the rate of opercular breathing movements as related to temperature. Cessation or change in rate of opercular movement is taken as

a criterion of response to stimulation by acids. Rate of response, as related to concentration of the acids is thus intimately associated with opercular rate, and the possibility existed that both rates were similarly affected by temperature changes. However, experiments designed to test that possibility have revealed that the temperature characteristics and the critical temperatures for the two processes are distinctly different. It was therefore concluded that the catenary reactions leading to chemical stimulation are independent of those processes governing breathing rhythm.

A study of the opercular movements of *Fundulus* as a function of temperature has brought out some significant facts which deserve consideration. The experimental set up for studying opercular rate is the same as that for chemical stimulation. At least an hour's adaptation time in the experimental dish passed before the time for ten gill movements of the fish was measured with a stop watch. At 0° and 1°C opercular movements practically cease, and no great reliance can be placed on data obtained at these temperatures. For the salt water tests one reading was taken on each of three fish and the average of the three used. Six fish were used in fresh water and an average taken. Such averaging is justified for data from individual fish treated separately gave essentially the same results as the averages from the several fish. An analysis of the data for the salt water tests showed that when log opercular rate was plotted against the reciprocal of the absolute temperature a linear band of points was obtained (Fig. 8). There is no apparent break in this relationship between the limits of temperature used. Although it does not show clearly from the plot, the opercular rate reaches a maximum and constant value at 25°C and above. The value of μ obtained from the slope of the parallel lines bounding the band of plotted points was found to be 8,400. There is a striking agreement between this value of μ and the value found by other investigators for similar respiratory movements.

The μ value has been thought to represent the "energy of activation" of the catalyst for the slowest process in the series of catenary events controlling the velocity of the phenomenon being studied (Crozier, 1924-25). Of course, with change of temperature or some other factor, a different process in the series may become the slowest, and μ would correspondingly change to a value typical of the catalyst for this new

reaction Certain processes such as O_2 consumption, and CO_2 production are common to all protoplasm, and it might be expected that such reactions might have similar catalysts, and hence similar values Such is the case, for certain phenomena dependent on cellular oxidation have typical oxidation temperature characteristics Perhaps the

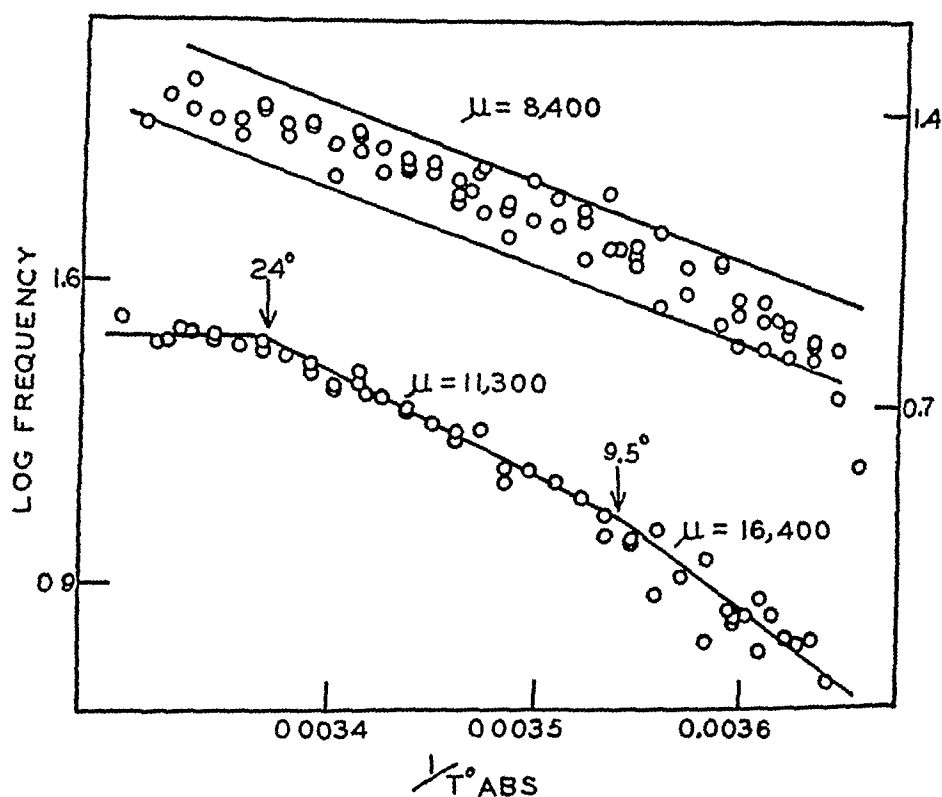


FIG 8 Log frequency (ten times the reciprocal of the time for ten movements) of rhythmic opercular movements of *Fundulus* plotted against $\frac{1}{T}$ Upper curve Breathing rhythm in salt water Lower curve Breathing rhythm in fresh water

most common values which have been found for respiratory oxidations are 8,000, 11,000, and 16,000

Crozier and Stier (1924-25 b) suggested that the value 8,000 may reflect the velocity of synaptic processes concerned in respiratory movements of fishes and other vertebrates A value of around 8,000 has been reported several times for the rate of respiratory movements

Crozier and Stier (1924-25 a) reported the value of 8,600 for pharyngeal breathing in the frog. A few years later Cole and Allison (1929) confirmed that value for the frog. 8,600 was also reported for gill contractions in larval *Amblystoma* (Crozier and Stier, 1926-27). Navez (1930) found the value 8,200 for breathing rhythm of dogfish. By subjecting goldfish to a temperature of 25°C for 3 hours Crozier and Stier (1925-26) obtain the value 8,300 for gill rate instead of the usual 16,500. Various examples from the invertebrates also might be cited where a μ of about 8,000 is obtained for respiration.

The relationship between *Fundulus* opercular rate and temperature is distinctly different in fresh water from that in salt (Fig 8). Below 9.5°C the temperature characteristic is 16,400, above 9.5°C it is 11,300, at 24°C the gill rate becomes constant. A further indication that the two μ values, 11,000 and 16,000, are markedly distinct is the fact that the variability of gill rate for fresh water is very much greater below 9.5°C, than it is above this temperature. Apparently a change in environment from salt to fresh water has so altered the catenary series of events that the slowest process in the chain is no longer the reaction whose catalyst has a thermal increment of 8,400. The values obtained for *Fundulus* breathing rhythm in fresh water, 11,300 and 16,400, are both characteristic of respiratory biological oxidations. It is evident that the temperature characteristics for *Fundulus* opercular rhythm may be experimentally altered by changing the environment of the fish from salt to fresh water.

A few examples will be cited to show that the μ values, 11,000 and 16,000, are indicative of oxidative and respiratory phenomena. A value of roughly 11,000 has been found for respiration in *Sepia*, oxygen consumption in *Lupinus* (Tang 1931-32), oxidation in *Arbacia* eggs (Rubenstein and Gerard, 1933-34), and for pulsation frequency in "accessory hearts" of *Notonecta* (Crozier and Stier, 1926-27). The value of 11,500 has been associated with catalysis by the hydroxyl ion (Crozier, 1924-25). The value of roughly 16,000 has been found for CO₂ production by *Lupinus*, *Phaseolus* (Crozier and Navez, 1930-31), *Pisum*, and *Vicia faba* (Tang, 1931-32), for pulsation frequency in accessory hearts of *Notonecta*, for CO₂ production by nerve ganglia, for the reduction of methylene blue by bacteria, for the deoxygenation

of oxyhemoglobin by carbon monoxide, and for respiration in the dogfish (Navez, 1930) The value of roughly 16,000 is often definitely associated with iron catalysis (Crozier, 1924-25)

It is evident, therefore, that the temperature characteristics 8,000, 11,000, and 16,000 are definitely associated with biological oxidations, or with reactions which are limited by the velocity of cellular oxidations These three values represent distinct reactions in the oxidative metabolism of protoplasm However, these may be catenary reactions (Crozier and Stier, 1924-25 *b*) and it might be predicted that under altered experimental conditions the organism would exhibit a corresponding change in thermal increment

Examples of such experimental modification of temperature characteristics have been furnished chiefly by the work of Crozier and Stier (1924-25 *b*) The temperature characteristic for pharyngeal breathing movements of the frog is 8,600 8 days after decerebration the μ value was definitely altered to 11,000 In grasshoppers decapitation causes μ to change from 7,900 to 16,200 and 11,200 The typical value for goldfish opercular breathing rhythm is 16,500 (Crozier and Stier, 1924-25 *b*) Subjection of the fish to 3 hours at 25°C causes this value to change to 8,300 The temperature characteristic of the heart rate of *Limax* changes from 16,300 to 11,500 according to the season of the year A μ value of 11,500 for *Limax* heart rate was changed to 16,200 by feeding the slug sugar After 4 days the temperature characteristic reverts to its original value, therefore the effect is reversible

It is not surprising, therefore, that the thermal increment for opercular breathing rhythm of *Fundulus* changes when the environment is altered from salt to fresh water The μ value changes from 8,400 over the whole temperature range in salt water to 16,400 below 9.5°C, and to 11,300 above this temperature in fresh water.

SUMMARY

1 Chemical stimulation as a function of temperature was studied by using oxalic acid in fresh and salt water and malonic acid in salt water as stimulating agents on *Fundulus* According to the Arrhenius equation the following μ values were obtained for the various acid solutions between 0 and 29°C for 0.002N oxalic in fresh water—

15,800, 33,000, for 0.0008N oxalic in fresh water—15,800, 33,000, 48,000, for 0.002N oxalic in salt water—19,400, 24,100, 56,500, for 0.004N and 0.002N malonic in salt water—20,600, 65,000. At a critical temperature there is a sharp transition from one thermal increment to another.

2 The chemical processes controlling stimulation do not change with concentration, for different normalities of a single acid yield the same μ values. Distinctly different temperature characteristics were obtained for stimulation by oxalic in salt and fresh water. Likewise stimulation by oxalic and malonic in salt water yielded very different increments. This temperature study indicates that the controlling chemical reactions determining rate of response are different for the same acid in two different environments, or for two dibasic acids in the same environment. Other work indicates, however, that the fundamental stimulation system is the same for all the acids in both environments. Chemical rather than physical processes limit the rate of response since all the values are above 15,000. Stimulation depends upon a series of interrelated chemical reactions, each with its own temperature characteristic. Under varying conditions (e.g. change of temperature, environment, or acid) different chemical reactions may become the slowest or controlling process which determines the rate of response.

3 The variation of response, as measured by the probable error of the mean response time of the fish, is the same function of temperature as reaction time itself. Hence variability is not independent of reaction time and is controlled by the same catenary series of events which determine rate of response to stimulation.

4 Breathing rhythm of *Fundulus* as related to temperature was studied in both salt and fresh water. In salt water the temperature characteristic is 8,400 while in fresh water it is 16,400 below 9.5°C, and 11,300 above this critical temperature. These μ values are typical of those which have been reported by other workers for respiratory and oxidative biological phenomena. A change in thermal increment with an alteration in environment indicates that different chemical reactions with characteristic velocity constants are controlling the breathing rhythm in salt and fresh water.

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EFFECT OF PROTEINS ON ELECTROPHORETIC MOBILITY AND SEDIMENTATION VELOCITY OF RED CELLS

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Since the increased sinking velocity of red cells seen in pregnancy and in various diseases is due to an aggregation of the cells, the question of the mechanism of the increased rate of sinking becomes that of the factors promoting aggregation, the influence of changes in cell or plasma specific gravity and in plasma viscosity is usually negligible.

The problem which presented itself may be stated as follows. The aggregation and therefore the sinking velocity of red cells are increased in human plasmas high in fibrinogen, as in pregnancy and in various febrile and neoplastic disorders. Aggregation and sinking velocity are increased by pure fibrinogen solutions and by various other agents, as gelatin, gum acacia, and casein, serum globulin is much less effective than fibrinogen and albumin almost without effect. Sinking velocity is proportional to the degree of aggregation of the cells as determined microscopically. The aggregation brought about by fibrinogen or gelatin is a rouleau formation and not, as in specific agglutination, a sticking at the first point of contact. The principal differences between slowly and rapidly settling human bloods are in the plasma, since the cells of a non pregnant woman sink almost as fast in "pregnant plasma" as do those of a pregnant woman, while pregnant and non pregnant cells both sink slowly in non pregnant plasma. The possibility of a difference in the cells of slowly and rapidly settling human bloods has not been excluded, we have demonstrated such differences in the cells of different species. The cells of horse blood settle many times as fast as those of beef blood, al

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though horse plasma contains no more fibrinogen than does cow plasma.¹ Since the cell concentration is a very important factor in determining settling rate, the sinking velocity increasing rapidly as cell concentration decreases, any quantitative work must be done with a constant ratio of cell to medium volume. Most of the above facts are reported by Fåhræus (1921).

The problem has been clearly stated by Fåhræus (1929), who says,² "The problem seems to hinge upon the manner in which the globulin increase changes the interface between the corpuscles and the plasma or, let us say, the surface of the red cells. Is it by increasing the surface tension, or by reducing the negative electric charge of the corpuscles or finally by depriving their surface colloids of adsorbed water?"

Most of the previously reported views on the mechanism of increased sedimentation velocity demand the formation of an adsorption coat of protein on the red cell surfaces, which should act by lowering surface charge or increasing surface tension. The first question to be answered, therefore, is whether such an adsorption coat is formed. Earlier evidence purporting to demonstrate this is unsatisfactory. Thus, an important line of evidence advanced has been that the isoelectric point of red cells is that of some plasma protein. Netter (1925) concludes that beef red cells carry an adsorption layer of albumin, horse cells of globulin. For a discussion of the sources of error in isoelectric point determinations see Abramson (1930), who concludes that the isoelectric point of normal red cells cannot be measured, that the isoelectric points observed are merely those of protein coats adsorbed on *damaged* cells, and that normal red cells do not adsorb any of the proteins investigated on the alkaline side of the isoelectric point and do not adsorb gelatin even when the gelatin is positively charged. We fully confirm Abramson on all of these points. These remarks do not apply, of course, to the reactions between specific antibodies and cell antigens, which are adsorption processes.

Another line of evidence is that of Wohlsch (1924), which is used

¹ We have not seen any report, previous to our work, of the settling of horse cells in beef plasma and *vice versa*.

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by Bendien, Neuberg, and Snapper (1932) in an attempt to explain the action of fibrinogen on sedimentation velocity Wohlisch and Wohlisch and Bohnen (1924) report microscopic evidence of elastic fibers passing between red cells, which they interpret as evidence of a layer of "denatured fibrinogen" or fibrin on the cells, causing the cells to stick together They have no evidence that these fibers are fibrin or that a fibrin coat exists but merely assume its existence in order to retain the Höber-Mond (1922) theory^{*} The fact that rouleau formation consists in a sliding of cells into such position that the minimum surface is exposed proves, as Fåhræus has pointed out, that a mere stickiness is not involved (in contrast to specific agglutination) This fact does not appear to have been adequately appreciated (Ponder, 1926, Abramson, 1934⁴)

Rothe (1924) emphasizes increase of interfacial tension as the principal factor in promoting aggregation and sinking velocity, assuming a fibrinogen adsorption coat He gives no experiments but argues by exclusion of other factors

Abramson first presented evidence against the presence of an adsorption coat He showed (1929) that repeated washing of red cells does not change their electrophoretic mobility, this means either that there is no adsorption coat or that it cannot be washed off Abramson (1930) further showed (his Fig 2) that the mobility of washed red cells in diluted serum is at no pH (above 3.5) the same as that of quartz particles in the same medium, this means either that there is no protein adsorption coat on the cells, or that the red cells selectively adsorb a protein having a different mobility from that adsorbed on the glass He further showed (1934) that the mobility of horse red cells in oxalated horse plasma is about the same as in horse serum, this means either that the red cells do not adsorb fibrinogen or that they already had a fibrinogen coat before being transferred to the serum More convincing evidence is his finding (1930) that while the isoelectric point of normal red cells cannot be measured, it is certainly lower than that of any plasma protein, while this does

^{*} The evidence quoted from Ley (1922) that the maximum agglutination of red cells in fibrinogen solutions is at pH 5.86 is not convincing, since Ley's cells were undoubtedly damaged by washing

⁴ P 260

not exclude the adsorption of small amounts of protein, it proves that a complete coat is not formed. Again, Abramson (1929) found that the mobility of pregnant human cells in their own plasma is normal, which demonstrates that the increased sinking velocity of the pregnant blood is not due to an adsorption coat of fibrinogen. Abramson's suggestion that the increased sinking velocity may be due to the adsorption of amounts of fibrinogen too small to be detected electrophoretically, but sufficient to make the cell surface more sticky is incompatible with the finding referred to above, that agglutination by rouleau formation is a surface tension phenomenon and not explicable by an increased stickiness.

EXPERIMENTAL

We may first consider our experiments designed to answer the question of a protein adsorption coat on the cells. These fall into two groups, one based on a comparison of the isoelectric point of the cells in protein solutions and the other based on determinations of the effect of added protein on the electrophoretic mobility of the cells. Electrophoretic determinations were made in a cylindrical cell of the Mattson (1928) type, the readings being taken 0.147 diameter from the top of the cell. The applied voltage was 9.1 volts per cm, the 232 volt power line being used. Each mobility figure is the average of five observations with each direction of current, with reversal between consecutive observations. Change in velocity, due to drift, on reversal of current, was significant only with low mobilities. When a difference does occur the average velocity for the two directions must be taken, rather than calculating velocity from the average time in the two directions. Temperature was noted and correction for viscosity made, in all cases the temperature was close to 25°C.

Viscosity determinations were made with the Bingham (1922) viscometer in a water bath at $25^{\circ} \pm 0.02$, using pressures from 60 to 300 cm of water, with transfer times from 90 to 440 seconds. The gelatin stock sols showed a slight component of plasticity since the observed viscosities at pressures of about 60 cm of water were a few per cent higher than with pressures of 300 cm of water. The values obtained with the high pressures were used for correcting the mobilities. We confirm Abramson (1928) in finding that the plasticity

does not hinder mobility at the voltages employed, since the same mobility was observed with 4.55 as with 9.1 volts per cm. The viscosity determinations were easily reproducible in our hands to ± 0.2 per cent.

In the first group of experiments we found that within 2 minutes after adding fresh, unwashed human cells to solutions of gelatin or plasma dilutions in M/50 phthalate buffer of pH 4.0 + 3 per cent glucose + 0.3 per cent NaCl, the cells were still negatively charged although the proteins were positively charged.⁵ However, the cells gradually reverse their sign of charge upon standing in the acid solution. Cells washed in M/50 buffer + 5 per cent glucose reverse their

TABLE I

Electrophoretic mobility of gelatin-coated glass particles and of washed cow and dog red cells in 0.04 per cent gelatin as a function of pH. The cells were washed 5 times in M/50 buffer pH 7.4 + 5 per cent glucose. The gelatin was dissolved in M/50 phthalate buffer + 5 per cent glucose. Readings made within 2 minutes after addition of cells to buffer mixtures.

pH	Glass	Cow	Dog
	$\mu/\text{sec} / \text{v} / \text{cm}$	$\mu/\text{sec} / \text{v} / \text{cm}$	$\mu/\text{sec} / \text{v} / \text{cm}$
7.4	0.92	1.90	2.30
6.0	0.88	1.86	2.35
5.0	0.48	1.65	1.72
4.6	0.21	1.25	0.91
4.0	+0.27	+0.26	+0.27

charge immediately when placed in protein solutions on the acid side of the protein isoelectric point. Thus, dog cells washed repeatedly in M/50 phosphate buffer, pH 7.4 + 5 per cent glucose, were immediately reversed when transferred to a solution of cow fibrinogen in M/10 buffer pH 5.0 (isoelectric point of fibrinogen at pH 5.6, determined electrophoretically), dog, cow, horse, and human cells similarly washed were immediately reversed in gelatin solutions or plasma dilutions at pH 4.0. The results shown in Table I are typical of the behavior of cells washed in such glucose-dilute buffer solutions. We interpret these results as meaning that normal red cells do not

⁵ This test of adsorption could not be applied to casein, since at pH 4.0 insufficient casein dissolves in the buffer to coat completely even glass particles.

adsorb proteins even when the latter are positively charged. Washing with 5 per cent glucose, however, or allowing the cells to stand in acid solution damages the cell surface, such damaged cells may then adsorb protein. Cells washed in 0.9 per cent NaCl are less likely to be damaged than those washed in sugar-buffer, but cells allowed to stand in any solution, even their own plasma, sometimes show abnormalities both in electrophoresis and in sedimentation velocity. For this reason we have confined our later experiments to unwashed cells from oxalated blood not more than 2 hours old.

TABLE II

Corrected electrophoretic mobility of protein-coated glass particles as compared with that of fresh, unwashed horse red cells. Egg albumin, casein, and gelatin were dissolved in M/50 phosphate buffer pH 7.4 + 3 per cent glucose + 0.3 per cent NaCl, horse fibrinogen in M/10 phosphate buffer pH 7.4. Albumin was dialyzed.

Protein	Concentration	Relative viscosity	Mobility		Corrected mobility (mobility \times viscosity)		Ratio Horse cells Glass
			Glass	Horse cells	Glass	Horse cells	
	per cent		$\mu/\text{sec}/\eta/\text{cm}$	$\mu/\text{sec}/\eta/\text{cm}$			
Egg albumin	0.01	1.07	0.75	1.50	0.80	1.61	2.01
	3	1.24	0.69	1.50	0.85	1.86	2.19
Casein	0.01	1.07	1.54	1.56	1.72	1.67	0.97
	3	1.99	1.24	1.62	2.47	3.08	1.25
Gelatin	0.01	1.07	0.71	1.62	0.76	1.73	2.28
	2	6.38	0.45	1.27	2.87	7.72	2.69
Horse fibrinogen	0.01	1.01	0.82	1.47	0.83	1.49	1.80
	1.6	1.44	0.64	1.49	0.92	2.15	2.34

In the second group of experiments, we find that at pH 7.4 the mobility of fresh, unwashed horse, beef, human, and dog cells in M/50 phosphate buffer + 3 per cent glucose + 0.3 per cent NaCl is unchanged on addition of several hundredths per cent of gelatin, casein, egg albumin, or homologous fibrinogen, more than sufficient to coat glass particles completely. Since in no case was the mobility of the cells changed by the protein it seems unnecessary to present tables of data. While this indicates that an adsorption coat is not formed at these low concentrations of protein, the possibility re-

mained that at the higher concentrations required to increase the sinking velocity of the cells, an adsorption coat is formed. Therefore the mobilities of glass particles and of fresh, unwashed horse, beef, human, and dog cells were compared also in 3 per cent casein, 3 per cent egg albumin, 2 per cent gelatin, and 1.6 per cent fibrinogen, all brought to pH 7.4, with a final electrolyte concentration of $M/50$. The ratio of the mobility of the cells to that of the protein-coated glass particles was always at least as high in the concentrated protein solutions as in the dilute. The results of a typical experiment are shown in Table II. These results demonstrate that the proteins investigated are not adsorbed by the red cells even in concentrated solutions. All theories of increased sinking velocity based on changes in surface tension or charge brought about by adsorbed protein must therefore be discarded, the proteins act without being adsorbed.

We may now consider the third possibility mentioned by Fåhræus, namely, that the proteins may act "by depriving their (the cells') surface colloids of adsorbed water." Such surface dehydration should lower the suspension stability of the cells. Actual evidence for such surface hydration has not heretofore been presented. In investigating this point on protein sols we find that observed mobility of gelatin and casein decreases with increasing protein concentration less than bulk viscosity increases. This must mean that double layer viscosity increases less than does bulk viscosity, since the electrolyte medium remains constant, charge density and double layer thickness are unchanged. The changes in mobility and viscosity with egg albumin are too small to permit a definite conclusion as to whether or not double layer viscosity remains the same as bulk viscosity.

It is difficult to visualize a protein molecule inside a layer only a few $m\mu$ thick, as is the case with $M/50$ to $M/15$ solutions, the concentrations employed in this protein work. Furthermore, electrical forces should prevent adjacent protein particles from approaching each other closely enough for either to be within the limits of the other's double layer. Nevertheless, it is seen that added protein does have some influence on double layer viscosity, since observed mobility decreases somewhat. The suggestion is therefore made that the decrease in observed mobility, which means increase in double layer viscosity, observed on increasing the protein concentra-

tion, takes place in some way without the added protein actually entering the double layer. An ability of a protein particle to orient water molecules within a zone extending beyond the limits of its double layer could account for this effect. This means that a given protein particle possesses a layer of oriented water molecules extending beyond this double layer and is thus able to influence the viscosity at the surfaces of its neighbors. This argument applies to the protein of the medium, with the protein-covered glass particles the surface of the particle under observation is therefore also hydrated, since its surface is the same as the added protein.

In the blood work, however, the particle under observation is a red cell, having a surface different from the added protein. A decrease in its observed mobility less than the increase in bulk viscosity of medium with increasing protein concentration cannot on the basis of the above argument be taken as evidence that the original surface of the cell was hydrated.

But if the cell surface were not hydrated, added gelatin or casein should effect an orientation of water molecules about the added protein particles, the effect extending to the originally unoriented water molecules in the cell's double layer. The double layer viscosity of the cell would increase even more than is the case with protein particles (since the double layer viscosity with the latter was already above normal before the additional protein was added) and the observed mobility of the red cell would decrease even more with increasing protein concentration than is the case with the protein particles themselves.

If, on the other hand, the observed mobility of the cells is but little decreased on increasing protein concentration, this may be taken to indicate that the viscosity of the cell's double layer was already high (due to orientation of water molecules) and is therefore but little increased by the action of the protein. Such a finding would therefore indicate that the red cell is normally surrounded by a layer of oriented water molecules. It therefore becomes desirable to determine how the electrophoretic mobility of red cells is influenced by added protein. Ponder (1926) found corrected mobility of human red cells greater in plasma than in 0.85 per cent NaCl. He ascribed this to a greater charge on the cells in plasma than in saline, without

considering the possibility that double layer viscosity differed from bulk viscosity. Since he does not give his viscosity data one cannot tell whether or not observed mobility decreased.

We have measured the electrophoretic mobility of fresh, unwashed horse, beef, human, and dog red cells in various protein solutions and have found that their observed mobility is unchanged or only slightly decreased from that in the protein free medium. We interpret this as meaning that viscosity of the cell double layer does not increase, or only slightly, with greatly increasing bulk viscosity. This is compatible with the view that the cell surface is hydrated. Since our protein solutions differed from the protein free medium only in that protein was present, there is no reason to believe that cell charge was altered in the various solutions.

The sedimentation experiments were carried out in glass tubes of 3.5 mm. bore and 35 cm. long. Fresh, unwashed cells were used, a constant ratio of 1 volume of cells to 2 volumes of protein solution being maintained in all experiments. After considerable experimentation to find a medium suitable for both sedimentation and electrophoresis observations the protein solutions were finally made up in $M/50$ phosphate buffer + 3 per cent glucose + 0.3 per cent NaCl at pH 7.4. The thoroughly mixed cell suspensions were sucked up into the glass tubes to form columns 30 cm. long. The tubes bore short segments of rubber tubing on their lower ends, which, after filling, were closed with spring clips.

The ash content of the undried gelatin was 1.0 per cent, of the casein 1.1, and of the egg albumin 5.9 per cent. Any correction for the salt effect on mobility here must be very rough. If one assumes that the ash represents an equal weight of NaCl we have, in terms of molar electrolyte carried into the solutions by the proteins in Table III, 0.03 M with albumin, 0.006 M with casein, 0.003 M with 2 per cent gelatin, and 0.0015 M with 1 per cent gelatin. The molar concentration of electrolyte due to the $M/50$ buffer and 0.3 per cent NaCl constantly present is about 0.07 M . The concentration of electrolyte due to salt content of the protein is thus increased by about 50 per cent with the albumin and about 10 per cent with the casein; the change in salt concentration with the gelatin is negligible. Since κ varies as the square root of concentration (Müller 1933), κ is increased, and mobility decreased, by about 22 per cent with the albumin and 5 per cent with the casein. The column headed "mobility corrected for salt effect" designates what the observed mobility would have been if the proteins had been salt free. This figure multiplied by bulk viscosity gives the 'corrected mobility' of the 8th column.

The findings on fresh unwashed cells are shown in Table III. It is evident that with the cells such as horse, human, and dog, which

TABLE III

Mobility and sinking velocity of fresh, unwashed (except where otherwise specified) red cells All solutions made up in M/50 buffer + 3 per cent glucose + 0.3 per cent NaCl, pH 7.4

Medium	Relative viscosity	Mobility	Mobility corrected for salt effect	Sinking for min indicated			Corrected mobility (mobility \times viscosity)	Corrected sinking (sinking in 30 min \times viscosity)
				10	20	30		
		$\mu/\text{sec } f/\text{cm}$	$\mu/\text{sec } f/\text{cm}$	mm	mm	mm		
Dog 1								
Buffer	1.07	1.60	1.60	0	0	0	1.71	0
3 per cent albumin	1.24	1.27	1.55	0	0	0	1.92	0
3 per cent casein	1.99	1.39	1.46	18	47	77	2.90	153
2 per cent gelatin	6.38	1.32	1.32	40	102	139	8.43	887
Dog 2								
Buffer	1.07	1.59	1.59	0	0	0	1.70	0
3 per cent albumin	1.24	1.32	1.61	0	0	0	2.00	0
3 per cent casein	1.78	1.40	1.47	27	85	134	2.62	238
1 per cent gelatin	3.58	1.43	1.43	10	53	115	5.12	412
Human 1								
Buffer	1.07	1.44	1.44	0	0	0	1.54	0
3 per cent albumin	1.24	1.13	1.38	0	0	0	1.71	0
3 per cent casein	1.99	1.19	1.25	6	23	39	2.49	78
1 per cent gelatin	3.58	1.29	1.29	12	40	73	4.62	261
Human 2								
Buffer	1.07	1.36	1.36			0	1.46	0
3 per cent albumin	1.24	1.09	1.33			0	1.65	0
3 per cent casein	1.99	1.13	1.19			10	2.37	20
1 per cent gelatin	3.58	1.13	1.13			21	4.05	75
Beef								
Buffer	1.07	1.04	1.04			0	1.11	0
3 per cent albumin	1.24	0.88	1.07			0	1.38	0
3 per cent casein	1.78	0.92	0.97			0	1.73	0
1 per cent gelatin	3.58	0.83	0.83			0	2.97	0
Horse 1								
Buffer	1.07	1.44	1.44	0	0	0	1.54	0
3 per cent albumin	1.24	1.18	1.44	0	0	0	1.79	0
3 per cent casein	1.78	1.50	1.57	51	118	135	2.80	239
1 per cent gelatin	3.58	1.43	1.43	22	64	97	5.12	347
Horse 1 washed in M/50 buffer pH 7.4 + 5 per cent glucose								
Buffer	1.07	1.33	1.33			0	1.45	0
3 per cent casein	1.78	1.24	1.30			1	2.32	2
1 per cent gelatin	3.58	1.29	1.29			1	4.70	4

can be aggregated and made to settle rapidly by added protein, gelatin is the most effective in increasing sinking velocity, with casein next, and albumin practically without effect. Beef cells do not settle at all in the 30 minute period in any of the proteins investigated. We have also found that beef cells will not sink more than 1 mm in an hour in either beef or horse plasma while horse cells sink 100 mm or more in 20 minutes in either horse or beef plasma.

DISCUSSION

In this paper data have been presented which are interpreted as indicating that red cell surfaces are hydrated. The establishment of a surface hydration for the red cells is a necessary but not a sufficient condition for the acceptance of the third possibility of Fåhræus. The remaining step is to show that certain proteins, as gelatin, casein, and fibrinogen have a capacity to dehydrate the surfaces, not possessed by albumin. We have not been able to devise any positive independent test of such dehydrating action. The only evidence that they do act in this way is the fact that the red cells are aggregated by gelatin and casein and not by albumin. The viewpoint may be briefly recapitulated as follows. All types of red cells investigated possess a layer of oriented water molecules extending beyond the limits of the double layer. Added gelatin, casein, or fibrinogen abstracts the water from the outer shell in the case of those red cells which are aggregated by these proteins but is unable to do so in the case of the red cells, as beef, which are not aggregated, presumably because the latter cells hold the water more tenaciously. This disorientation, *i.e.* with respect to the cell, does not, however, extend into the double layer since double layer viscosity, as indicated by the electrophoretic findings, is but little changed by added protein. The surface energy at the interface of the cell and its medium is greater the thinner the oriented water layer, the increased surface tension holding the less hydrated cells together.

SUMMARY

The isoelectric point of normal red cells cannot be measured but is certainly lower than that of any plasma protein. Red cells are easily damaged so that they will adsorb proteins from low concentra-

tions Normal red cells do not adsorb protein even from concentrated solutions, as is evidenced by the finding that the ratio of the mobility of the cells to that of the proteins themselves is at least as high in concentrated casein, albumin, gelatin, or fibrinogen solutions as in dilute

The finding that the observed mobility of red cells is unchanged or only slightly decreased when bulk viscosity is increased by added protein is interpreted as indicating that the red cell surfaces are hydrated The aggregating effect of certain proteins has been determined and is assumed to be due to their dehydrating effect on the cells Some types of cells, as beef, are not aggregated, presumably because they are resistant to this dehydrating effect The difference in the behavior of different types of red cells demonstrates the importance of the nature of the cell as well as of the medium in determining the rate of aggregation and therefore of sedimentation

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ELECTROKINETIC PHENOMENA

XII ELECTROOSMOTIC AND ELECTROPHORETIC MOBILITIES OF PROTEIN SURFACES IN DILUTE SALT SOLUTIONS

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INTRODUCTION

It has been shown by Abramson (1) and confirmed by Daniel (2) that the ζ -potential of inert microscopic particles covered by protein films is the same as that calculated for the ζ potential of the wall of the electrophoresis cell when coated with the same protein. These measurements had usually, though not always, been made in the presence of considerable concentrations of electrolytes. The significance of these results may also be stated as indicating that the ratio of the electroosmotic mobility, u , to the electrophoretic mobility, v , was found to be 1.0, so that,

$$R = \frac{u}{v} = 1.0,$$

very nearly, taking the same sign as the ζ potential.

White, Monaghan, and Urban (3) have recently contended that the value of R for protein covered surfaces is equal to 1.0 only when the electrolyte concentration is greater than 0.01 M. They found that, with their technic, ratios up to 2.3 were obtained in very dilute salt solutions and distilled water. Contrary to these observations are the experiments of Bull (4). Bull compared the ζ potential calculated not only from electrophoresis and electroosmosis experiments but also from streaming potential measurements. Bull found that protein-covered surfaces in dilutions as great as 3.5×10^{-4} N yielded a value of R equal to 1.0 within the limit of error, for ζ de

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terminated by electrophoresis, electroosmosis, and the streaming potential technics White, Monaghan, and Urban's comparable figures between $R = 1.31$ and $R = 1.57$

In this communication, evidence will be presented which indicates that in dilute salt solutions certain protein-coated surfaces exhibit a value of R equal to 1.0, very nearly, thus confirming the experiments of Bull, and contrary to those of White, Monaghan, and Urban

Methods

Type of Cell—A flat electrophoresis cell (5) which has been described in detail as Cell "B" by Abramson and Grossman (6), and which had been checked against another cell, was employed in these investigations The use of this cell, which is quite rectangular in cross-section, provides a hydrodynamic system which follows closely the theory of von Smoluchowski It is necessary to clean the cell with particular care between experiments Since the entire apparatus is of one piece of glass, molar NaOH could be used to remove the adsorbed protein This was followed by dilute acid and distilled water

Calculation of the Field Strength—The values of the mobilities given in the figures have been calculated as usual from Ohm's law (7, 8) and the observed velocities Due correction was made for fluctuations of current and therefore changes in field strength within the cell We wish to emphasize that other methods of calculation of the field strength which do not entail the measurement of current may lead to erroneous conclusions This point is of special importance when comparing results obtained over a wide range of electrolyte concentrations

The use of plaster of Paris plugs (6, 9) at the junction of suspension and electrode vessel prevents convection of the electrolyte from the electrode vessel and diminishes the chance of contamination of the particles under investigation

Preparation of Suspensions—Finely powdered, carefully cleaned, quartz particles (9), about 1μ in diameter, were used to adsorb the proteins investigated In one experiment, protein-coated pyrex glass particles were simultaneously observed with protein-coated paraffin oil globules The particles were, as usual (10), suspended

in concentrated protein solutions (1 per cent) and the suspension then diluted to final concentrations of protein ranging between 0.2 per cent and 0.02 per cent. While the particles were in contact with the protein solution, the electrophoresis cell was filled with a protein solution of the same concentration. The cell itself was finally washed with a large volume of the final dilution of the suspension. Three proteins—Coignet gelatin, Agfa *Lichtfilter* gelatin, and a highly purified ghadin (Dill)—furnished the surface deposits of adsorbed materials.

The suspensions having the lowest conductances in Table II contained protein which had been dialyzed for several days in an ice box.

Recording of Time—One of us observed the motion of the particles in the electric field and pressed the stop watch lever when the particles had traversed a suitable distance, their direction being reversed during the measurement. The other observer recorded the stop watch readings which were unknown to the observer timing the particles.

EXPERIMENTAL

I Calculation of v and u Assuming $c = 0$

In view of the controversial nature of the problem under discussion, a large number of measurements were made at random at various levels of the electrophoresis cell in each experiment. It can be readily shown that velocity level curves found in a long and wide cell of rectangular cross section are given by an expression of the form,

$$y = b(x - x^2) + c \quad (1)$$

Here y is the velocity observed at a distance, x , from the ceiling of the cell with a total thickness, $x = 1$. The terms b and c are constants. When $R \approx 1.0$, $c = 0$. If $R = 1.5$ or $R = 2.0$,

$$y = (v - u) = -c \quad (2)$$

with proper regard to sign, at $x = 0$ and $x = 1$. The sign of c is always negative when $R > 1$.

Three to five measurements of y were usually made, in the experiments here reported, at each of the different levels. These values which generally agreed within 10 per cent were averaged and a value of b calculated (Table I) for each set of points at each level investi-

gated, letting $c = 0$. An average value of b was readily obtained from the points, as indicated in the table, and a theoretical curve drawn by means of equation (1), taking the average value of b . Values of b from which the theoretical curves were obtained lay between the limits $\alpha = 0.1$ and $\alpha = 0.9$. Evidently this curve corresponds to the condition that $R = 1$, for $u = v$. In addition, when desired, similar curves were drawn for $R = 1.5$ and $R = 2.0$, based

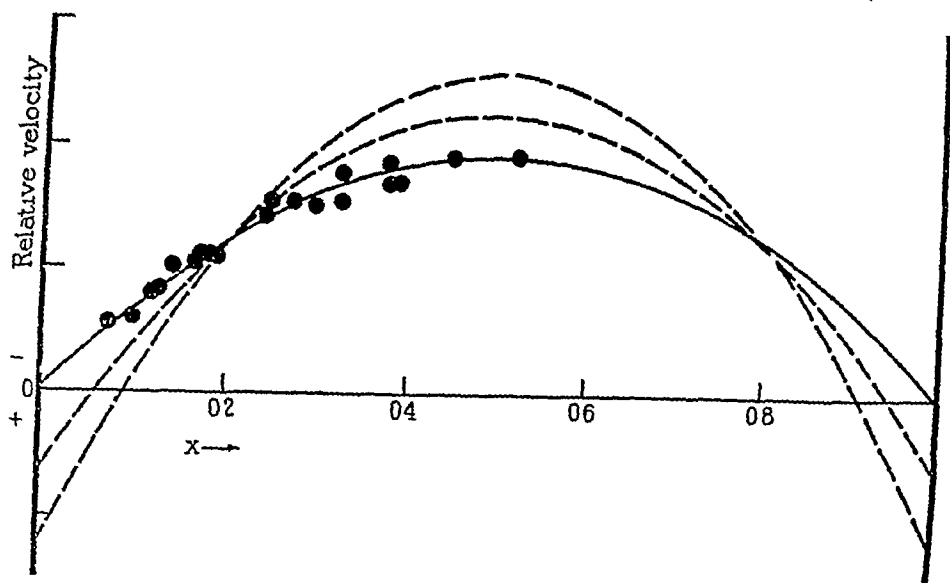


FIG. 1 Experiment with gelatin-coated (Coignet) quartz particles. Ordinate values of v are in relative units plotted against depth, x , in the electrophoresis cell. The smooth, unbroken curve (lowest) has been calculated from equation (1), taking $c = 0$. The two upper curves (dashed) have been calculated, taking $R = 1.5$ and $R = 2.0$. It is evident that within the limits of error the experimental points fit the curve for $R = 1.0$, indicating that electrophoresis and electroosmosis are equal. See Table II. The velocities given in each figure have been reduced to constant field strength.

upon the value of v obtained at the level 0.2(0.8) with appropriate values of c . Evidently the value of v must remain the same, independent of the value assigned to R . In consequence, curves derived from v held constant but with u varying, must all intersect at the level 0.2(0.8). This procedure avoids the errors¹ or difficulties

¹ It can be easily shown that in the determination of R by the use of Equation 3 an error of 10 per cent in the measurement of $v_{0.5}$ or $v_{0.2(0.8)}$ causes a divergence of about 30 per cent in the value of R .

inherent in using the equations,

$$u = 2(v_0 \xi - v_{0.2}(0.8)) \quad (3)$$

and

$$u = v_0 - v_{0.2}(0.8), \quad (4)$$

where the subscripts refer to the cell depth. Our present procedure, rather, takes into consideration a large number of experimental points and includes electric mobilities throughout the entire cell

The results of our experiments and calculations are presented in Figs 1-7 and in Tables I and II. Every point, rather than averages

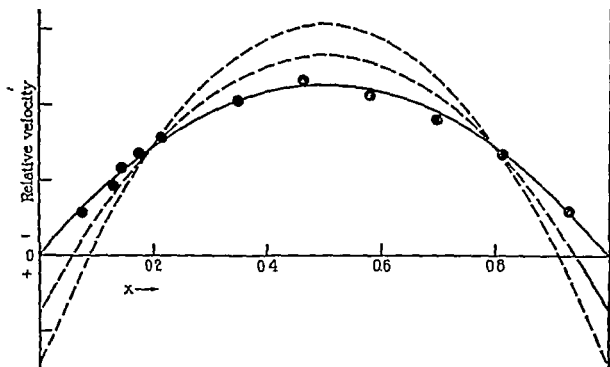


FIG 2 Similar to Fig 1, except that each point is an average of several measurements at the levels indicated. See Table II

at a given level, is plotted in Figs 1 and 5. Note in the figures that the experimental values in every case fit the theoretical curve drawn on the assumption that $c = 0$. This agreement demonstrates that under our conditions the electrophoretic mobility of particles coated with gelatin (and, in one experiment, with gliadin²) is practically equal

² Our results with gelatin and with gliadin do not appear to be in accord with experiments of Kemp and Rideal (11). These investigators reported that the mobility of gliadin-coated particles of different radii is dependent upon their radii in dilute solutions.

to the electroosmotic mobility even in the most dilute solutions. The value of R , in accord with these experiments, is, therefore, very close to 1.0. Certainly there is no indication that the data fit the dashed curves drawn so that $R = 1.5$ or $R = 2.0$. Occasionally, very slight movement in the reverse direction was noted at the floor of the cell (discussed in more detail below) as depicted in Figs. 3, 4, 5, and 7. The velocity in the reverse direction was, however, very small and when this reversal was observed, the reversal occurs very

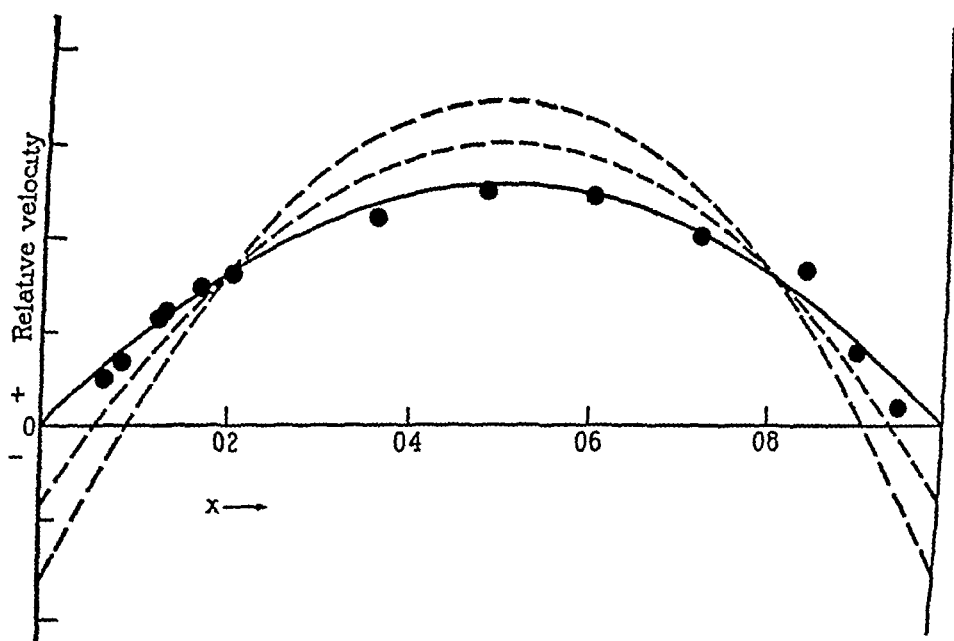


FIG. 3 Experiment with gladin in 50 per cent ethyl alcohol, otherwise similar to Fig. 1, except that each point is an average of several measurements at the levels indicated. See Table II.

close to the wall, indeed. The interpretation of this behavior is rather difficult. With Henry (12), who states, "It was neither possible nor desirable to make observations right against the wall, but the velocity which should obtain there was deduced by the extrapolation of observations made at various small distances from the wall," we ascribe reversals tentatively to the "wall effects" discussed by Stimson and Jeffrey (13), Lens (14), Bull and Moyer (15), Bikerman (16), and Abramson (17).

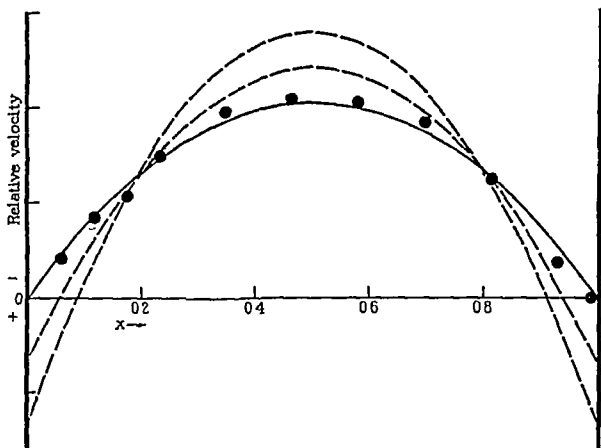


FIG 4 Experiment with gelatin-coated (Agfa) quartz particles otherwise similar to Fig 1, except that each point is an average of several measurements at the levels indicated See Table II

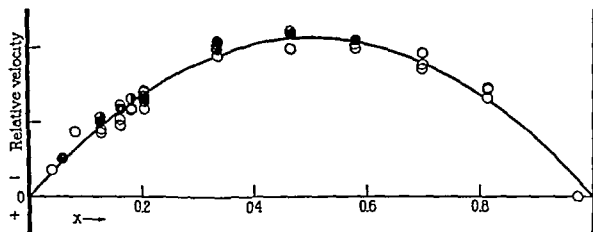


FIG 5 Experiment with gelatin-coated (Agfa) pyrex glass and paraffin oil particles each point being a single observation. The open circles are glass particles, the closed circles are paraffin oil droplets The smooth curve has been drawn for $c = 0$ The agreement indicates that R is very close to 10 See Table II

II Determination of v and u by Linear Extrapolation

To exclude wall effects and obtain a value of the electric mobility at the wall ($x = 0$, $x = 1$), use was made of the fact that equation (1)

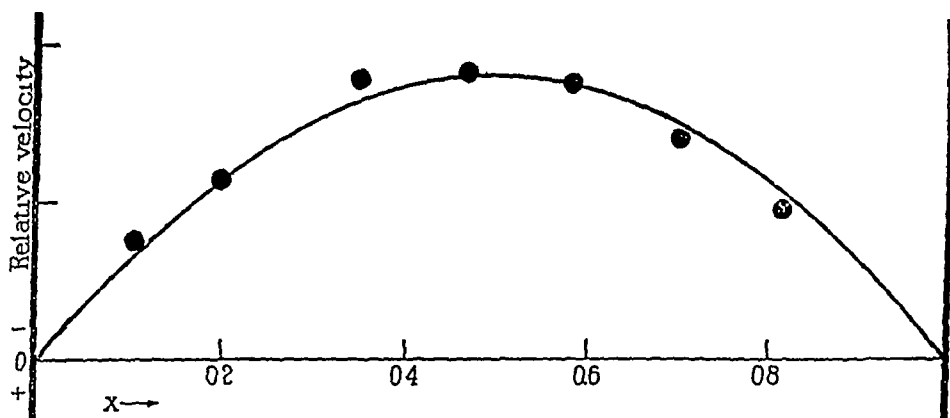


FIG 6 A control experiment with $m/100$ KCl The smooth curve has been drawn for $c = 0$ Note the similarity between this curve and all the other figures in the paper See Table II

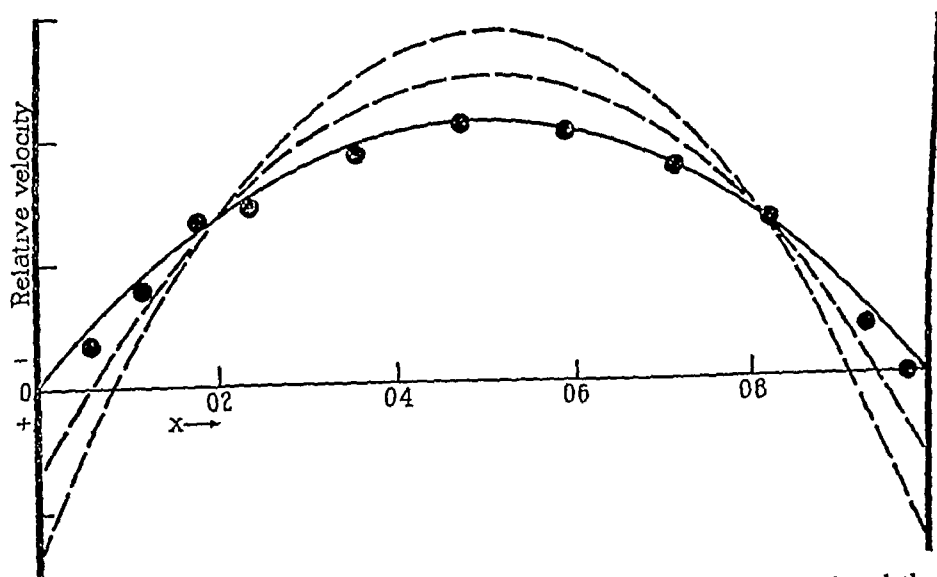


FIG 7 Similar to Fig 1 except that Agfa gelatin has been employed and the points represent averaged measurements See Table II

can be employed in a linear form That is, the observed mobilities, y ,

$$y = b(x - x^2) + c,$$

can be plotted against $(x - x^2)$ The slope of the line is evidently equal to b and its intercept with the ordinate gives c Our data, plotted in this way, could readily be fitted by straight lines The

TABLE I

Values of b for the different values of x , calculated by assuming that $c = 0$ Note the constancy of b in each case, this indicates that the intercept is negligible and that $R = 1.0$ very nearly

Fig 1		Fig 2		Fig 4		Fig 6	
x	b	x	b	x	b	x	b
0.118	0.77	0.131	0.82	0.119	0.81	0.105	0.40
0.125	0.77	0.144	0.94	0.174	0.74	0.197	0.36
0.141	0.84	0.186	0.90	0.233	0.83	0.318	0.40
0.165	0.76	0.214	0.92	0.348	0.86	0.465	0.37
0.169	0.80	0.349	0.91	0.465	0.84	0.580	0.36
0.177	0.78	0.465	0.93	0.581	0.84	0.698	0.34
0.181	0.76	0.581	0.87	0.698	0.87	0.814	0.32
0.188	0.72	0.698	0.86	0.814	0.82		
0.240	0.78	0.813	0.88				
0.247	0.83						
0.271	0.78	Fig 3		Fig 5		Fig 7	
0.294	0.73	x	b	x	b	x	b
0.325	0.81	0.126	0.52	0.128	0.84	0.116	1.5
0.325	0.71	0.132	0.53	0.163	0.80	0.175	1.8
0.376	0.80	0.168	0.52	0.182	0.87	0.233	1.6
0.388	0.72	0.204	0.50	0.205	0.79	0.350	1.6
0.447	0.78	0.360	0.48	0.205	0.83	0.465	1.7
0.518	0.78	0.480	0.51	0.336	0.88	0.582	1.7
		0.600	0.52	0.465	0.86	0.698	1.7
		0.720	0.51	0.580	0.84	0.815	1.8
		0.840	0.56	0.697	0.86		
				0.813	0.92		

straight line giving the best fit, in each case, was extrapolated to $(x - x^2) = 0$ in order to obtain c The values of c so obtained furnish a method of testing our value for R independent of the first method In Table III these values of c are listed, all are very close to the origin or at it

TABLE II
Characteristics of the Systems Investigated

Fig	Particle	Flat surface	Adsorbed protein	Medium	Final protein concentration	Specific conductance of suspension
					per cent	mhos $\times 10^3$
1	Quartz	Soft glass of cell	Gelatin (Coignet)	Distilled water	0.2	5.1
2	Quartz	Soft glass of cell	Gelatin (Coignet)	Distilled water	0.2	5.1
3	Quartz	Soft glass of cell	Gladin	50 per cent ethyl alcohol	0.2	1.6
4	Quartz	Soft glass of cell	Gelatin (Agfa)	Distilled water	0.05	2.1
5	Pyrex and paraffin	Soft glass of cell	Gelatin (Agfa)	Distilled water	0.2	9.2*
6	Quartz	Soft glass of cell	Gelatin (Agfa)	0.01 M KCl	0.2	153
7	Quartz	Soft glass of cell	Gelatin (Agfa)	Distilled water	0.02	0.39

* This high conductance is presumably due to the presence of dissolved glass

TABLE III

The ratio, R , of electroosmotic mobility, u , to electrophoretic mobility, v , in the systems investigated. Values of c have been obtained by a linear extrapolation of the curves of η plotted against $(\alpha - \alpha^2)$. These values of c represent an extrapolated rather than an assumed value. It is of interest to point out that this method of plotting permits the calculation of the mobility of the particles at $\alpha = 0$ and $\alpha = 1$, with avoidance of the questionable wall effect described in the text. C , v , and u are in relative units.

Fig	c	v	u	$R = \frac{v+c}{v}$
1	0	0.123	-0.123	1.00
2	0.002	0.142	-0.140	0.98
3	-0.004	0.0805	-0.0845	1.05
4	-0.009	0.212	-0.221	1.04
5	0	0.156	-0.156	1.00
6	-0.004	0.062	-0.066	1.07
7	-0.031	0.289	-0.320	1.11

Having obtained c by means of this method, R can readily be evaluated by means of equation (2). The values of v were obtained at the level 0.2 (0.8) from the straight lines just described. These lines represent the "stationary levels," according to Komagata (18), for a cell of dimensions corresponding to our cell (6). Values of R thus calculated are also given in Table III. The values of R range from 0.98 to 1.11 and show no tendency for R to increase as the specific conductance decreases. Note that the specific conductances of the suspensions are listed in Table II. The specific conductance here provides more information in regard to electrolyte concentration than "salt added." In dilute solutions, the salt added may contribute less to the ionic strength than that contributed by the salt of the protein solutions, even though dilute.

DISCUSSION

White, Monaghan, and Urban have explained their results by invoking a "polarization" of the double layer on the particle and claim that electrophoretic measurements in dilute solutions would have to be corrected for this retarding effect to yield true values. In the light of our data, any polarization of the double layer either affects the protein coating of the wall and the particle to an equal extent, or else it retards the particle so slightly that it cannot be detected at present. Since the electroosmosis past a plane protein coated surface is identical with the electrophoresis of very small particles coated with the same protein, certainly in solutions as dilute as those investigated here (where κr would be small (10)), it follows that these data are consistent with the suggestion previously advanced (10) that the protein molecule "takes its own radius with it" on adsorption and hence keeps κr the same for both the wall and the particle.

SUMMARY

The ratio of electroosmotic to electrophoretic mobility of certain protein coated surfaces is very close to 1.0, even in very dilute solutions of electrolytes.

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THE STRUCTURE OF THE ULTRAVIOLET ABSORPTION SPECTRA OF CERTAIN PROTEINS AND AMINO ACIDS*

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The ultraviolet absorption of the proteins in the region above 2500 Å comprises a broad band with its maximum about 2800 Å. The spectrophotometric studies of Judd, Lewis (1), Smith (2), and Smith and Marrack (3) have indicated the main quantitative features of this band without, however, disclosing evidence of finer structure. Indeed, with the exception of Spiegel, Adolf and Krumpel (4), who were able to distinguish several maxima in the absorption band of serum albumin, those investigators who have used the arc between metallic electrodes, in air, as the source of radiation have not discovered any details of structure. Vlès and Prager (5) on the other hand found a band system consisting of nine narrow bands in serum albumin, when using the hydrogen discharge tube, which emits a continuous spectrum in the ultraviolet. With such a tube as source, Ross (6) has observed a similar resolution in the absorption bands of gelatin and several other proteins, and Lavin and Northrop (7) have found narrow bands in the absorption of crystalline pepsin.

The ultraviolet absorption of the amino acids has likewise been extensively investigated. Although the aliphatic amino acids do not show appreciable absorption above 2500 Å, the aromatic amino acids, tyrosin, tryptophan, and phenylalanine give absorption in the same spectral region as the proteins, as shown by Dhéré (8), Ward (9), Smith (2), Gróh and Hanák (10) and others. The absorption band of the proteins has in consequence been generally attributed to the content of these amino acids.

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The existence of narrow component bands in the absorption of the aromatic amino acids has been demonstrated as with the proteins only with the use of a source which gives a continuous spectrum Stenstrom and Reinhard (11), with the under-water spark, and Ross, and Lavin and Northrop, with the hydrogen discharge tube have found two bands in both tyrosin and tryptophan in the region 2700–2900 Å Ward, who used nickel electrodes in air, reported a group of bands in phenylalanine Although Smith could not confirm this, Ross found five, and Lavin and Northrop found six narrow bands lying between 2400 and 2700 Å in this amino acid Ross noted the correspondence in position of these bands with the band elements of several proteins

EXPERIMENTAL

The primary object of the present work was the discovery of the spectral characteristics which may be associated, in serum protein, with immunologic activity It was necessary first to provide a background against which might be discerned any peculiarities associated with biological activity This we have attempted to do by investigating a number of familiar and important proteins Independent observations were also made on the aromatic amino acids and a number of other amino acids

The proteins¹ studied and the methods of their preparation were as follows

1 Serum albumin, from horse serum Crystallized three times after removal of the globulin, as described by Adair and Robinson (12), and dialyzed free from sulfate

2 Crystalline egg albumin Prepared according to Heidelberger (13) and crystallized three times

3 Serum pseudoglobulin, and

4 Euglobulin The precipitate obtained from normal horse serum on half saturation with ammonium sulfate was separated and reprecipitated twice by the same procedure The solution of protein was then dialyzed free from salt The resulting precipitate represented the fraction which we have considered as euglobulin, while the protein

¹ The authors wish to thank Dr Michael Heidelberger of the Department of Medicine for several of the proteins used in this work

which remained in solution after dialysis yielded the pseudoglobulin fraction

5 Thyroglobulin, from the hog Prepared according to Heidelberger and Palmer (14)

6 Crystalline insulin Supplied to us by Dr Oskar Wintersteiner of the Department of Biochemistry

7 Pneumococcus Type I antibody Prepared by the method of Felton (15) from immune horse serum Two preparations (B76 and B77) were used These contained about 65 per cent specifically precipitable nitrogen as determined by the quantitative precipitin reaction with Type I acetyl polysaccharide

The amino acids studied were the following tyrosin, cystine, tryptophan, phenylalanine, and histidine, the two latter supplied to us by the Department of Biochemistry, and proline and oxyproline kindly supplied by Dr Randolph West of the Department of Medicine All had been purified by recrystallization

Solutions of the different proteins and amino acids were made up to contain 5 mg of the test substance per cubic centimeter Progressive dilutions were then made, the concentration in each successive step being one half that in the preceding Spectrographic exposures were made on a single photographic plate of each series of dilutions, the time of exposure and the width of the slit, for each series, being kept constant The slit openings used were between 0.050 and 0.100 mm In these series it was possible in many instances to distinguish narrow absorption bands that could not be made out in any single exposure The spectrograms of the series of graded dilutions also disclosed differences, in each substance in the relative intensities of the narrow bands

The solutions were maintained at pH 7.0 throughout the work by the use of $\kappa/10$ phosphate buffer for the primary solutions and for all of the subsequent dilutions In the cases of tyrosin and insulin, however, the substances were dissolved in 0.01 κ HCl on account of their low solubility at pH 7

The temperature of the solutions at the moment of exposure was that of the room (21–26 C) In a few experiments glycerol was used as the solvent and the exposure made when the solution had been brought to a low temperature by immersing the fused quartz cell in which it was contained in liquid air The technical difficulties of such low temperature work are very great, however as Lavin and Northrop have pointed out in a paper which appeared subsequent to the completion of our experiments Almost immediately after solidification the glycerin begins to become opaque in consequence of "cracking" and it becomes impossible to admit sufficient light to affect the photographic plate The information obtained from the low temperature experiments was meager and is not included in the data presented in Table I and Fig 2

The spectrograph which was used was one of the Littrow type, and was constructed by one of us (C B C). The single quartz lens is 56 mm in diameter with a focal length for $\lambda 2536$ of 450 mm. The spectrum given by this lens is of sufficient length to give a distinct separation of the fine absorption bands without the loss of contrast which occurs when a lens of greater focal length is used. This lens proved somewhat more satisfactory than one of shorter focus, however, so that the selection of the focal length stated cannot be regarded as fortuitous. The lens was "figured" by zonal polishing for the correction of longitudinal spherical aberration, and during this process was tested by monochromatic light (Hg 5461) provided by light from the mercury arc passing through the optical system of the spectrograph itself. Exposures were made on $3\frac{1}{2} \times 4\frac{1}{2}$ Wratten M plates. That portion of the spectrum which is in sharp focus at a given setting of prism and lens, is short, with a single lens of large relative aperture and of high degree of aspheric correction, and with our lens did not exceed 80 mm, so that plates of larger size would not have been justified. The Hg arc spectrum was photographed on each plate immediately adjacent to the first and last exposures of each absorption series.

The source of radiation was an hydrogen discharge tube operated on 4400 volts. The capillary portion of the tube was water-cooled, its dimensions were 6 mm inside diameter by 250 mm length. The crystal quartz end-window was sealed on with hard deKhotinsky cement. The tube was alternately exhausted, with a Hyvac pump through a sealed-on P_2O_5 trap and filled with hydrogen through a palladium thimble until the lines of atomic hydrogen disappeared in the region 2000–3000 Å. We are greatly indebted to Prof. Harold Urey of the Department of Chemistry of this University for invaluable assistance in the design and construction of the hydrogen tube.

A quartz condenser was used to focus the radiation from the hydrogen tube upon the slit of the spectrograph. During this adjustment a fluorescent screen was used to indicate the convergence of the ultraviolet rays upon the slit, if visible light is brought to a focus at the slit, the illumination of the latter by ultraviolet radiation is very likely to be found far from uniform along its length.

The narrow bands which can be distinguished in the ultraviolet absorption band of the proteins and amino acids which we have investigated vary considerably in distinctness. While some are prominent, others are exceedingly faint and can be made out only under certain conditions of illumination and visual acuity. The measurement of the position of many of the narrow bands is quite impossible with the usual type of micrometer microscope, since the magnification reduces the visual contrast too greatly, and it becomes impossible to locate the center of a band. We have been able, however, to surmount to a considerable extent the difficulties of measurement by equipping the stage of the micrometer microscope with a long cross-hair, which extends across the full width of the plate, and is carried on a slide so that it may be moved over the plate to a position directly above the center of an absorption band. This maneuver is made with the free hand while the plate is under observation by the unaided eye. The plate is first

moved out of the optic axis of the microscope to a position where it can be readily observed and the substage mirror adjusted to give the optimum intensity and direction of illumination for the detection of the narrow bands. The desired portion of the plate is now brought into the field of the microscope and the stage moved longitudinally by means of the micrometer screw until the long cross hair, which has not been disturbed after being placed over an absorption band, is brought into coincidence with the cross hair in the ocular. The Hg lines on the

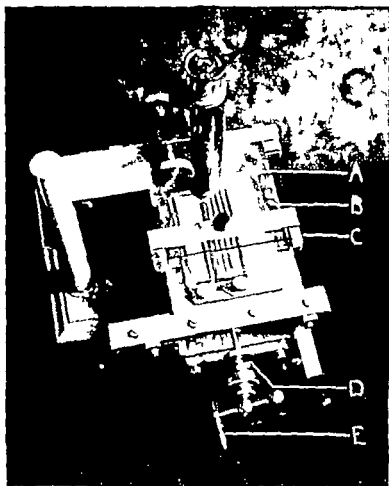


FIG. 1. Micrometer microscope with long cross hair. *A* stage, *B* guide for rotation of plate-carrier, *C* movable slide with cross hair, *D* milled head for rack and pinion motion, *E* crank for micrometer screw.

plate serve as reference lines from which the wavelengths of the absorption bands can be calculated by means of the Hartmann formula.

The complete micrometer microscope which was constructed by one of us, (C B C) is shown in the photograph Fig. 1. The plate carrier which rests upon the stage has a mechanism by which it can be rotated about its center for orientation of the plate. The stage is movable transversely by rack and pinion at right angles to the direction of longitudinal motion which is controlled by the

micrometer screw A human hair was used as the cross-hair, this was adjusted to lie parallel with the direction of transverse motion of the stage

Ten or more settings of the long cross-hair were made in the measurement of each of the narrow bands The differences between individual readings were naturally greater with faint than with strong bands With the latter duplicate readings were frequently made to within 1 Å Even with the faintest bands the error of measurement probably does not exceed 5 Å Several plates of each of the test substances were examined The average values of wavelength for the bands which could be distinguished in the various substances are given in Table I

TABLE I

Table of Wavelengths of Narrow Bands in Proteins and Aromatic Amino Acids

Serum albumin				2533	2583	2613	2645		2688	2733	2788	2847	2900
Egg albumin				2532	2587		2650		2680	2742	2799	2855	2923
Thyroglobulin				2534	2581	2614	2645		2682	2743	2796	2841	2909
Euglobulin			2487	2529		2616	2640		2680	2749	2795	2849	2915
Pseudoglobulin					2587		2649		2691	2747	2794	2849	2916
Pneumococcus anti-body					2591		2649		2685	2768		2850	2911
Gelatin				2529	2584		2644		2679	2745		2839	
Insulin				2530	2586		2645		2683	2766		2839	2898
Tyrosin									2672	2747		2816	
Tryptophan									2694		2794		2888
Phenylalanine	2366	2418	2466	2517	2574	2606	2635	2671	2714				

It is evident in this that among the proteins the positions of the narrow bands correspond with one another very closely Vlcs and Prager noted this relation with serum albumin, egg albumin, and casein Bands at certain positions are present in some of the proteins while apparently wanting in others Even if the error in measurement is as great as 5 Å, it seems clear that certain bands depart considerably from the "group" position, as for example the band at 2766 Å of insulin, and that at 2855 of egg albumin

The relative intensities of the narrow bands are indicated in the curves, Fig 2, which were traced by a recording microphotometer We are indebted to Dr Garman of Washington Square College for his kindness in making these records The curves do not reveal the presence of numerous bands which can be distinguished on direct examination, but show those that appear most distinct visually Since the various solutions to which the microphotometer records correspond

were of different molalities, the heights of the curves may not be used to compare the amounts of absorption at a particular wavelength given by the different substances. The curves do give, however, a measure of the relative intensities of the more distinct bands within the spectrum of a given substance.

It is evident on examination of these curves, as well as on direct examination of the absorption series spectrograms, that the proteins differ among themselves in the relative intensities of the narrow bands. The differences are sufficient to give each absorption curve a characteristic configuration.

The narrow bands show with great distinctness in serum albumin (Curve 1, Fig. 2). They are somewhat more sharply defined in our curve than in that of Vlès and Prager, who made their spectrograms at 0°C, while ours were made at 21–26°C. No tracing was made for egg albumin, its absorption, however, in its fine structure resembles that of serum albumin. The narrow bands appear almost uniformly distinct in thyroglobulin also, but the absorption curve as a whole shows a close resemblance only to that of euglobulin, from which it can hardly be distinguished except for the prominence in the latter of the band at 2680 Å. The intensity of this band varies, however, in different spectrograms of euglobulin. In several it has appeared more intense than the broad band which has its peak at 2795 Å.

In the curve of pseudoglobulin the broad band about 2794 Å is relatively more intense than in the preceding proteins. The narrow bands, however, are less distinct than in these, this fact is less evident in the photometric curve (Curve 4, Fig. 2) than in the spectrograms themselves.

Except in the region of the strongest band (2700–2850 Å) the curve for the Pneumococcus Type I antibody runs almost exactly parallel with that of pseudoglobulin. The suggestion given by this resemblance that this antibody is a modified pseudoglobulin is supported by the recent observations of Chow and Goebel (16), which leave little room for doubt that the pneumococcus antibody is carried by the pseudoglobulin fraction and not as was earlier supposed, by the euglobulin of serum. These authors have found that antibody shows chemical differences from normal pseudoglobulin. We have found that there are likewise spectrographic differences: the total absorption

ULTRAVIOLET ABSORPTION SPECTRA

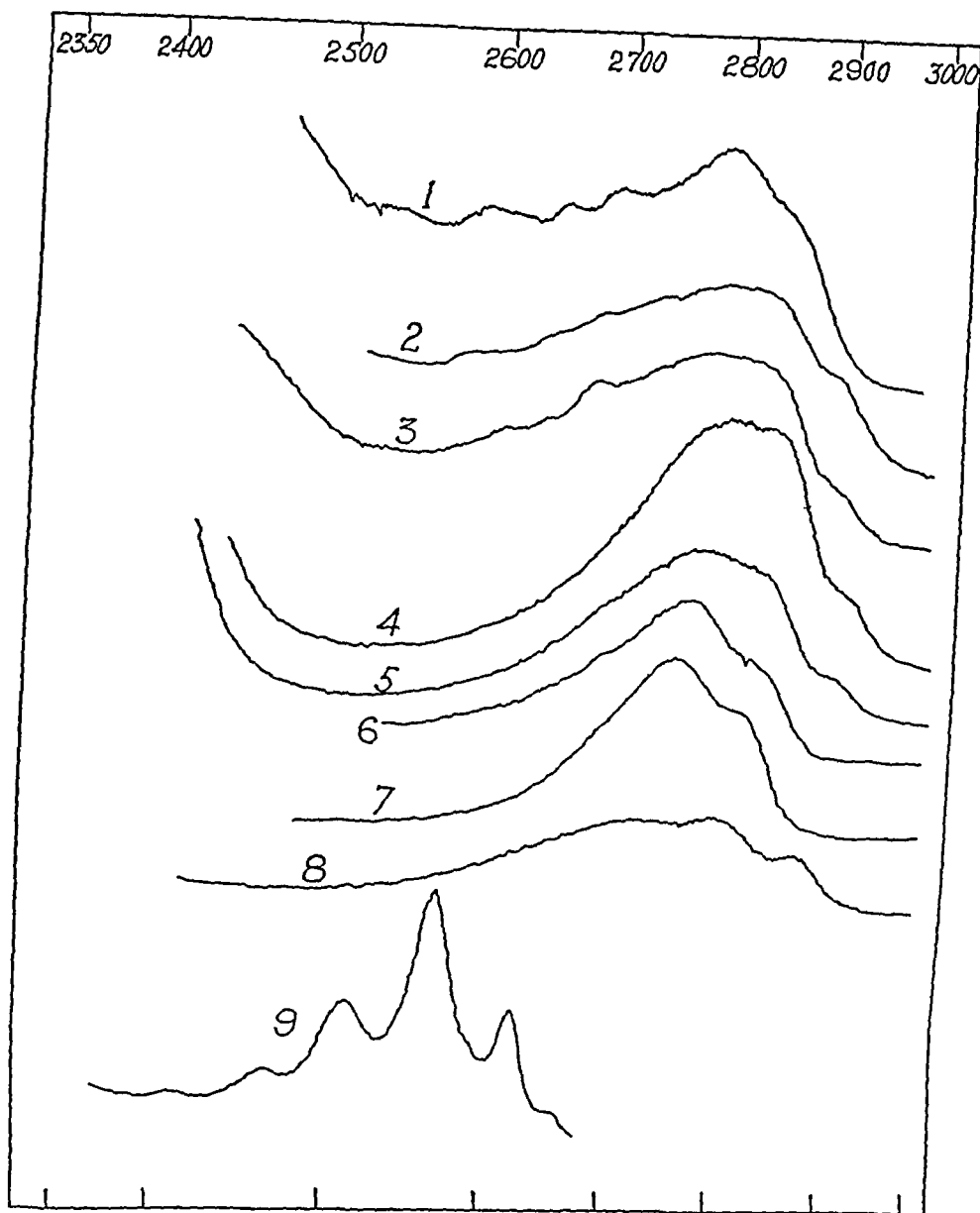


FIG 2 Microphotometric curves of ultraviolet absorption of proteins and aromatic amino acids

between 2700 and 2850 Å is relatively smaller in antibody than in the "normal" protein, and the point of maximum absorption is at 2768 Å rather than 2795 Å

No microphotometer tracing was made of gelatin It is evident

in the absorption series that the maximum of absorption is at 2584 Å. The broad band about 2795, and the band about 2900, which are found in most of the proteins, are wanting. We were able to distinguish clearly in gelatin the four narrow bands found by Ross and attributed by him to phenylalanine, as well as two other bands at longer wavelengths.

The curve for insulin (at pH 4, Curve 6, Fig. 2) shows the resemblance to that of tyrosin which Kuhn, Eyer, and Freudenberg (17) have noted for this protein. Neither their absorption curves, however, nor those of Graubner (18) show the narrow band structure which is evident in our spectrograms as four narrow bands on the short wave side of the peak at 2766 Å. Some of these are faintly indicated by points of inflection in the curves given by the latter author.

Among the amino acids, we have found no evidence of absorption of wavelengths longer than 2200 Å by proline or oxyproline. The sharply delimited end absorption of histidine comprises a broad band which has its maximum about 1950 Å. It gives no indication of resolution into components. Cystine shows in addition to end absorption, which extends with concentrated solutions to 2450 Å, a band with its maximum about 2490 Å. This appears on our plates much less distinct than one would be led to expect from the descriptions of Foster, Anslow, and Barnés (19). It could not be resolved.

The relatively sharp and narrow band of tryptophan (Curve 8, Fig. 2) which lies on the long wavelength side of the broad central region of absorption has its center according to our measurements at 2888 Å. The values of 2875 and 2900 Å have been given by Stenstrom and Reinhard, and by Ross, respectively. We find the center of the absorption maximum of this amino acid at 2795 Å. This band can be distinguished clearly from one almost equally strong with maximum at 2694 Å. When these two bands are not distinguished as separate, the absorption maximum appears about 2750, as found by Stenstrom and Reinhard, Ross, and Lavin and Northrop. The maximum of absorption by tryptophan has been given moreover by Gróh and Hanák as 2775, by Ward as 2800, and by Smith as 2790. The values of the two latter observers agree closely with ours, their method appears to have given the position of the absorption maximum accurately, although it did not reveal the lesser bands. The band of tryptophan which Smith

has described at 2180 Å and which appears on Ward's curve is evident on our plates, but is not resolved

The absorption maximum of tyrosin (Curve 7, Fig 2) in solution at pH 2.2, is found on our plates at 2747 Å. All observers have agreed on the position of this band. The relatively narrow band which we find at 2816 has been observed only by those who have used a source giving a continuous spectrum. Several observers (Dhéré, Stenstrom and Reinhard, and Gróh and Hanák) have noted that the spectral absorption of tyrosin is affected by the pH, the maximum of absorption lying farther toward the red in alkaline than in acid solutions. It seems probable that this shift is due to change in position and intensity of the narrow band just described. In our curve at pH 2.2 and in that of Stenstrom and Reinhard at pH 4 this band is small and lies near 2800. In alkaline solution (pH not stated) Ross found it at 2840, together with the band at 2750. Gróh and Hanák at pH 11.0 and Stenstrom and Reinhard at pH 12.7 (incorrectly quoted by Ross) found only one large band near 2900. There is no evidence in these cases that the band at 2750 had disappeared and it seems probable from study of the curve of Stenstrom and Reinhard that it was still present but obscured by the more intense band at 2900. A third band of low intensity is evident on our plates at 2672. It can barely be made out on the photometric curve. The large band found by Smith at 2240 affords on our plates no indication of resolution.

In the spectrum of phenylalanine (Curve 9, Fig 2) we have found nine bands between 2366 Å and 2714 Å. Five of these correspond to the bands observed by Ross, but lie from 5 to 14 Å deeper in the ultraviolet than the values given by this observer. There appears to be somewhat closer agreement between our results and those of Lavin and Northrop as far as can be made out from their figure. It is evident from the photometric curve that the band at 2574 is the dominant one. The bands at 2366, 2606, and 2714 are feeble and do not appear in the reproduction although the two latter are evident in the original tracing.

DISCUSSION

The theory that the ultraviolet absorption of the proteins, in the region above 2500 Å, is due to the aromatic amino acids is supported by the observations reported here.

The narrow bands which appear between 2530 and 2690 in all of the proteins may be assigned with some confidence to phenylalanine, as Ross and Lavin and Northrop have previously suggested. These bands occupy slightly longer wavelengths in the proteins than in the amino acids (Table I). The average amount of this shift is 12 Å, within the limit of accuracy of measurement it appears to be the same for each of the bands and to be uniform throughout the proteins. The relative intensities of the bands are not the same, however, in the proteins as in the amino acids. The band at 2574 for example which is by far the strongest in phenylalanine is not more marked in the proteins than the bands corresponding to those at 2517 and 2635 of the amino acid. On the other hand the band about 2680 which is one of the strongest in serum albumin and euglobulin is represented in phenylalanine by a relatively feeble band.

Only a small part of the ultraviolet absorption of the proteins, in terms of amount rather than spectral extent, may be referred to phenylalanine. Much the larger part, which occurs between 2700 and 2850 to 2900, may be attributed to tryptophan and tyrosin.

The band of tryptophan at 2794 may be held responsible for the occurrence of the maximum of absorption at this wavelength in several of the proteins. The band of this amino acid at 2694 cannot be identified in the proteins and indeed is so broad that it cannot be distinguished readily in the amino acid itself. The sharp band at 2888 Å, however, corresponds to a band which appears between 2898 and 2923 in all of the proteins which have been examined except gelatin. This band was found by Lavin and Northrop in crystalline pepsin and attributed by them to tryptophan. In the proteins this band appears shifted by 10 to 35 Å toward the red from its position in the amino acid.

The absence from the gelatin spectrum of this band and the one at 2794 is clearly correlated with the lack of tryptophan in this protein (20).

The large band of tyrosin at 2747 gives origin apparently to the absorption maxima of insulin and pneumococcus antibody near 2766 Å. Tryptophan appears for this reason to have a smaller share in the absorption of these two proteins than it has in other proteins which contain this amino acid. This suggests that these two proteins have a

relatively small content of tryptophan, although it is not at all certain that the amount of spectral absorption is in general proportional to the content of amino acid

The band present in all of the proteins between 2839 and 2855 Å corresponds to the band which is found in tyrosin in acid solution at 2816, and in alkaline solution, at 2840 Å or longer wavelengths, depending on the pH. The band about 2850 is very prominent in all of the proteins. Its exact position is probably influenced, as in the case of the 2747 Å band of tyrosin, by its proximity to a band of the more strongly absorbing tryptophan, the adjacent band in this case being that at 2888, which would tend to shift the apparent maximum of the tyrosin band to a longer wavelength. In gelatin and insulin in which the tryptophan influence is small, this tyrosin band lies at 2839 Å, having thus a definitely shorter wavelength than in most of the other proteins.

The position of this band is of interest in connection with the theory of Stenstrom and Reinhard, who have sought to relate the shift in this band, in tyrosin itself, with change in pH, to the ionization at the —OH group which is attached directly to the benzene ring. An effect upon the ionization at this point results, they believe, in the protein molecule, from the proximity of this —OH group to the —NH₂ group of an adjacent amino acid. The pneumococcus antibody is known from the work of Felton (21) and Chow and Goebel to possess an increased basicity as compared with the normal pseudoglobulin. On the theory of Stenstrom and Reinhard this greater basicity should occasion an even wider displacement of this tyrosin band than in the normal protein. However, the tyrosin band has the same position in the pneumococcus antibody as in the normal pseudoglobulin. The increased basicity of the antibody is thus left unexplained, in view of the fact that Chow and Goebel have found no essential difference, beyond a slightly higher lysine content, in the distribution of the basic amino acids in antibody and normal protein.

The experiments at low temperatures were inconclusive. We have been led to expect, from our observations (22) on the metal-porphyrin compounds at the temperature of liquid air, marked changes in the relative intensities of the bands. We did not, however, observe such changes. Vlès and Prager on the other hand noted a change in the

relative intensity of one of the bands of serum albumin between 0° and 37°C

The magnitude of the displacements to longer wavelengths, which are observed in the narrow bands of the proteins, from their position in the amino acids,—from 10 to 35 Å—suggests that the effect of polypeptid linkage is not upon the electronic energy of the amino acid, but rather upon its vibrational energy

It might at first sight appear surprising that the aromatic amino acids should retain their spectral character to so great an extent when combined in the protein molecule. It may be remarked, however, that the parts of the amino acid molecule which are involved in polypeptid linkage are separated by two or more carbon atoms from the benzene ring. Arnold and Kistiakowsky (23) have found that the mutual influence upon their absorption spectra of two chromophore groups within a molecule extends through a carbon chain only when this is two atoms or less in length, the spectra being unaffected when the separation is greater than this.

CONCLUSIONS

1 The absorption spectra of a number of proteins in the region 2500 to 3000 Å have been found to comprise from six to nine narrow bands. In consequence of variation in the relative intensity of these bands from protein to protein, the absorption curve has a characteristic configuration for each protein.

2 These bands correspond closely in position with the narrow bands which appear in the absorption spectra of tryptophan, tyrosin, and phenylalanine. Tryptophan and tyrosin each present three bands, phenylalanine shows nine.

3 The bands in the proteins are accordingly attributed to these amino acids. In the proteins the bands are displaced from the positions which they occupy in the uncombined amino acids, in most instances, by 10 to 35 Å toward longer wavelengths.

4 The absorption spectrum of Pneumococcus Type I antibody resembles that of normal pseudoglobulin but shows characteristic differences.

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THE BACTERICIDAL EFFECT OF ULTRAVIOLET RADIATION ON *ESCHERICHIA COLI* IN LIQUID SUSPENSIONS

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I

INTRODUCTION

The effect of ultraviolet radiation on microorganisms has been studied frequently. Extensive material can be found in Hollaender (16), and in Duggar (8). The present paper describes a study of the lethal effects of measured quantities of monochromatic ultraviolet radiation on bacteria in liquid suspensions, as part of a program to obtain a more intimate knowledge of the reaction of microorganisms to radiant energy.

It has been customary to irradiate bacteria spread in presumably monocellular layers on agar surfaces. Certain sections of these agar surfaces are irradiated for different time intervals with ultraviolet (in later work (2, 6, 10, 11, 19) monochromatic ultraviolet) radiation. Other sections, protected against the radiation, serve as the controls. The effect has been determined by an estimate of the relative density of bacterial growth on the agar surfaces, or by an actual count of the colonies. This method has the advantage of permitting the easy handling of a large number of exposures, and the quick determination of percentage effects in a rough way. It has the disadvantage that one cannot be certain that he has a single layer of organisms on the agar surface, especially if the time of exposure is extended. Further, the percentage killing effect cannot be determined exactly, especially for the lower values, radiation effects on the agar may be pronounced, and it is not easily possible to follow other than lethal¹ effects produced.

¹ Lethal is interpreted in this communication as meaning "unable to form visible colonies." This does not exclude the possibility that certain functions of the organisms are continued after the ability to divide sufficiently to form visible—

by the radiation on the organisms. It is especially difficult to follow physiological effects.

For the latter reason and because we sought a method by which we could determine small percentage effects with higher precision, we further developed the liquid suspension method (1, 4, 5) described by Duggar and Hollaender (9). This method involves more elaborate apparatus, and makes more tedious the determination of the survival ratios. It necessitates the use of suspending materials which are non-absorbing for the wave lengths used in the experiments, and which are of such composition that the organisms will not clump in them, *i e*, that subsequent dilutions will produce an equivalent diminution in the concentration of bacteria. The method of exposure must be such that each organism is equally subject to the radiation, and the radiation which is scattered by the organisms must be accounted for. But with these problems solved, the method lends itself very well to the determination, not only of lethal effects, but also of effects, other than lethal, which may be produced.

II

Technique

The work described below was conducted exclusively² with a culture of *E. coli*. The growth curve of the organism was carefully determined at 32°C, and this temperature was maintained for all subsequent incubations. The cultures were transferred to agar slants daily at a definite hour, and were used for most of our work after 15 hours. We found that at this time the cultures showed the most desirable characteristics, *i e* lowest number of double cells (about 10 per cent), highest percentage of colony forming organisms (about 90 per cent), and ease of removing from agar. This we called our "standard" culture. "Young" cultures came from a 6 to 7 hour slant, and contained 95 per cent viable and about 70 per cent double cells. "Old" cultures came from slants 8 to 10 days old, kept after the first 24 hours at room temperature. They contained 5-10 per cent double cells and only 30-40 per cent viable organisms. It was possible to repeat the con-

colonies has been impaired. Besides the lethal effect produced by the radiation, we have observed effects produced by energies not sufficient to impair permanently the reproductive activity of the organisms. This material will be made the subject of further communication (17).

² A number of experiments were conducted with a culture of *S. marcescens*. Because of difficulties due to pigmentation and occasional clumping, especially after washing, the results will not be discussed in this report.

ditions of the standard cultures without difficulty, but the situation was more difficult with young and old cultures. The variation in viability was much greater, a factor which will be discussed later.

The organisms were washed off the agar slants with a physiological salt solution (3 gm NaCl, 0.2 gm KCl, 0.2 gm CaCl_2 , 1000 cc. distilled water), poured into a sterile culture tube, shaken thoroughly, and filtered six times through a heavy layer of absorbent cotton. The suspension was free of clumps etc. as shown by microscopic observation and dilution tests. The number of organisms was estimated from the number of test tubes used and the appearance of turbidity. Since all steps in the procedure were well standardized, the estimate was usually so accurate that only one dilution had to be poured when plating.

A number of experiments were conducted with more thoroughly washed bacteria. The suspension described above was centrifuged at about 2000 R.P.M. for 15 minutes, the supernatant liquid poured off, the precipitate stirred, shaken thoroughly in freshly added salt solution and recentrifuged. This process was repeated three times. After the last centrifuging the suspension was again filtered six times through a heavy layer of cotton. The standard organisms (15 hour) gave, after washing, about 70 per cent of colony forming organisms as determined by plate and microscope counts, and the old organisms about 60 to 70 per cent colony forming ones, indicating that repeated washing inactivated some organisms especially in a standard culture, and that in the old cultures dead bacteria were probably clumped through the centrifuging and removed by subsequent filtration. Washing was not successful with young bacteria, since it was not possible to centrifuge out a sufficient number of organisms in a reasonable time.

The process of irradiation was similar to that described in previous publications (9), but the apparatus was somewhat modified for this work. (For details see Fig. 1.) The exposure cells were also similar to those described previously, except that the windows were fastened by means of rubber gaskets which permitted more careful adjustment and cleaning of the quartz windows.

Each exposure cell was filled with 6 cc. of bacterial suspension containing 1 to 3×10^8 organisms per cubic centimeter in such a manner that it was certain that control and exposure cells contained the same concentration of organisms. The material in these cells was stirred rapidly for 10 minutes before the so called "zero control" was removed; that is, a typical experiment was performed, but without exposure, thus giving a good check on the actual exposure cell content. The material was removed from the cells in 0.1 cc. quantities at once, and diluted in our standard salt solution. The method and speed of removing this quantity was worked out carefully to insure that each 0.1 cc. contained a representative sample of the cell content. Both cells were held in a constant temperature tank at 18°C .

The material was stirred rapidly (at least 300 R.P.M.) while actual exposure was made. The method and speed of stirring is extremely important for the efficiency of the radiation effect. It was found, however, that increasing the speed of stirring above 300 R.P.M. did not further increase the efficiency of the radiation

The beam of ultraviolet radiation ($< 3000 \text{ \AA}$) which entered the exposure cell could be followed by the fluorescence it produced up to about $\frac{1}{4}$ or $\frac{1}{3}$ of the cell thickness. Within this distance (about 5 mm), the radiation was absorbed, or scattered and then absorbed by the dense suspension of organisms in the cell to such a degree that no radiation left it, except for a very small percentage scattered back into the beam.

The intensity of the beam was measured before and after each irradiation by means of a standardized high sensitivity thermopile and galvanometer. Each exposure lasted 2 to 5 minutes. After each exposure 0.1 cc of the material was removed and at once diluted $1:10^3$ with salt solution. The rest of the suspension

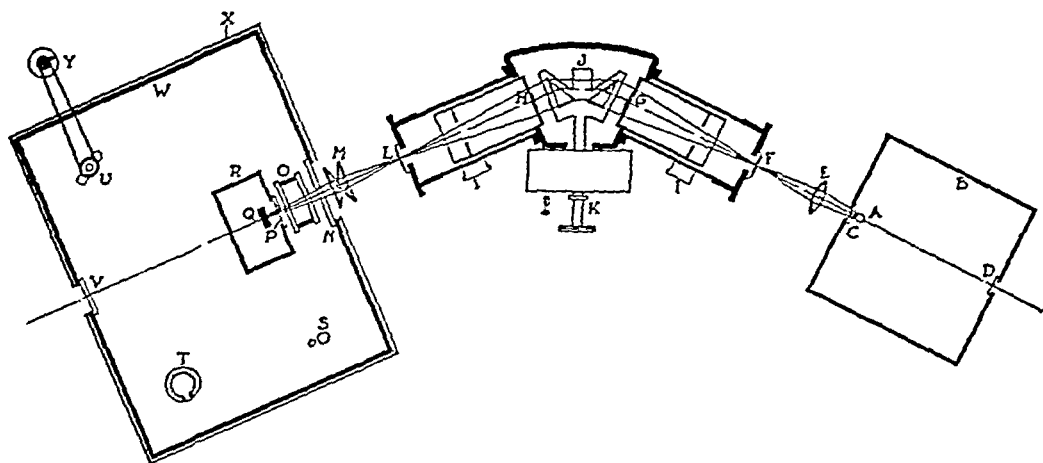


FIG 1 Diagram showing arrangement of apparatus for irradiating bacteria in liquid suspension with ultraviolet radiation

A, quartz capillary mercury vapor lamp (18), B, water tank, C, D, N, V, quartz windows, E, M, quartz condensing lenses, F to L, Bausch and Lomb quartz monochromator, O, exposure cell with quartz windows, P, Q, R, thermopile and case, S, thermal regulator, T, heating coil, U, sturrer, W, constant temperature exposure tank

was re-exposed and the procedure repeated until eight to ten exposures were obtained. After all the exposures had been made there was always 5 cc left in the exposure cell, a quantity still sufficient to fill the cell, so that no radiation was lost.

The dilution of the suspension was continued with 99 cc of salt solution until there were 100 to 200 colony forming bacteria per cubic centimeter. The material was plated out at once with nutrient agar. Counts were made after 48 hours, and all colonies on the plate were counted. The average of four plates was taken.

The entire procedure beginning with the removal of the bacteria from the agar slant to the pouring of the last agar plate usually did not take more than 40 minutes. Since the lag phase of these organisms at 18°C lasted more than 3 hours,

the procedure was safe. No difference in sensitivity could be found between organisms irradiated at once and those irradiated 40 minutes after removal from the agar slant. Special tests were also made to determine whether continuous exposure interrupted exposure caused by our method of irradiation, and also the interruption needed for the removal of suspension, could influence the energy needed to inactivate the organisms. Inside our limit of error, no difference could be found. No effect of the radiation on the suspending salt solution could be found. Neither was any effect observable when a suspension in which 99 per cent of the organisms had been killed was added to a fresh suspension of the same bacterial concentration. These tests eliminated the possibility of toxic effects due to the medium or to the previously affected organisms (3).

The situation with washed bacteria was less satisfactory. 50 to 60 minutes were required to wash these organisms. Including the time of irradiation usually 2 hours would have passed from the time of removal from the agar slant to the plating of the last culture. The washed bacteria tested at once after washing had not more than about 70 per cent colony forming organisms. Thus the data obtained with these washed organisms are not as reliable as those obtained with standard unwashed organisms. (All microscope counts were made with the Petroff-Hausser bacteria counter. The determinations were found not to be so reproducible as our plate counts. Only non-irradiated organisms were counted by this method.)

III

Calculations and Results

If a bacterial suspension is uniformly sensitive to radiation, the decrease, $-dN$, in the number, N , of viable organisms in the suspension when an increment of energy, dE , is applied will obviously be proportional to the number, N , of viable cells present, to the average fraction, μ , of the total energy absorbed by each bacterium, and of course inversely proportional to the energy, ϵ , required to inactivate a single bacterium. This may be expressed by the equation

$$-dN/dE = 1/\epsilon N\mu$$

If we represent by N_T the total number of bacteria, then for a very dense suspension in which practically no energy is lost by transmission or scattering

$$\mu N_T = 1$$

Hence

$$dN/dE = -1/\epsilon N 1/N_T$$

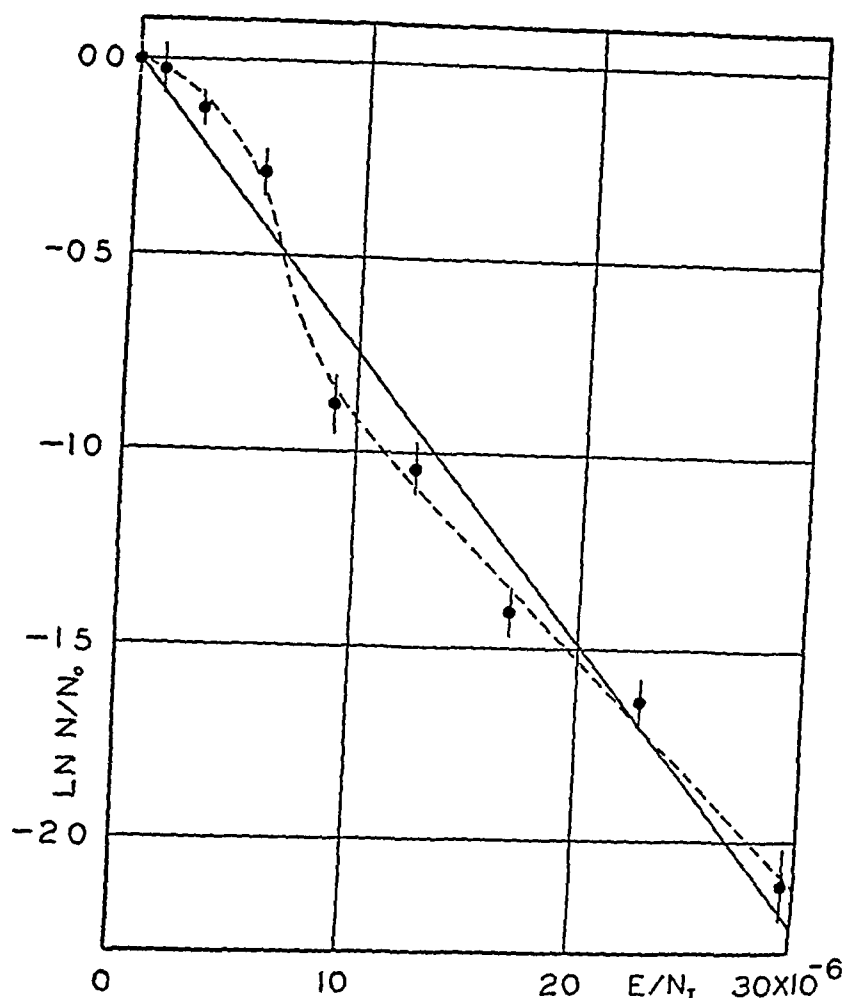


FIG 2 Graph of a typical experiment showing the logarithm of the survival ratio ($\ln N/N_0$) plotted against E/N_T for *E. coli* irradiated by $\lambda 2650 \text{ \AA}$. Probable errors are calculated separately for each point. The full curve is the best straight line through the points. The broken curve shows deviations from the logarithmic function from which the sensitivity distribution may be calculated.

Integrating, we obtain the survival ratio as

$$N/N_0 = e^{-\frac{E}{N_T} \frac{1}{\epsilon}}$$

where N_0 is the number of viable organisms at $E = 0$ (N_0 in general will be somewhat less than N_T).

This treatment is similar to that of a monomolecular reaction, with the sensitivity, $1/\epsilon$, taking somewhat the rôle of a velocity constant.

Such a treatment, however, is indicated by the experimental results, and because of its simplicity, one feels justified in placing a certain amount of confidence in the calculations. Attention should be called to the fact that the equation automatically corrects for absorption of energy by all non viable organisms in the suspension, and eliminates all need for consideration of the absorption coefficient of protoplasm for ultraviolet radiation when sufficiently dense suspensions of bacteria are used. It further provides a "natural" end point at which

TABLE I

Energy of Inactivation for E. coli Suspended in Salt Solution and Irradiated by $\lambda 2650 \text{ \AA}$

Culture	N_T (bacteria/cc.)	ϵ (ergs/bacterium)	Culture	N_T (bacteria/cc.)	ϵ (ergs/bacterium)
Standard (15 hour)	(1.26×10^8)	(30.4×10^{-4})	Old (10 day)	1.11×10^8	9.0×10^{-4}
	1.50×10^8	16.6		1.48	11.8
	1.35	13.3		3.10	3.3
	0.81	12.3		2.08	5.6
	1.11	11.9		4.42	5.5
	1.23	9.4	Old Washed	3.18×10^8	3.8×10^{-4}
	1.13	13.5		5.04	2.3
	1.62	14.1		4.74	5.2
	4.40	13.7	Young (7 hour)	2.8×10^8	31.1×10^{-4}
	Average	$13.1 \pm 0.5 \times 10^{-4}$		4.7	19.7
Standard Washed	2.48×10^8	7.3×10^{-4}		8.2	15.8
	3.62	4.3			
	4.40	6.8			
	Average	6.1×10^{-4}			

the energy, ϵ , required to inactivate one bacterium equals the average energy absorbed per bacterium, E/N_T . Usually, this point is arbitrarily taken for a survival ratio of 50 per cent, but here we see that if the survival ratio is an exponential function of the applied energy, when $\epsilon = E/N_T$, $N/N_0 = e^{-1} = 36.8$ per cent.

A graph of the natural logarithm of the survival ratio, N/N_0 , plotted against E/N_T will give a straight line, the slope of which is $-1/\epsilon$. A typical experiment is plotted in Fig. 2, indicating that to a first approximation at least, our observations may be treated according

TABLE II
Energy of Inactivation for E. coli As a Function of Wave Length

Wave length	ϵ (ergs/bacterium)					
	Hollaender and Claus	Gates (11)	Wyckoff (19)	Erismann and Noethling (10)	Coblentz and Fulton (6)	Henri (14)
2250 Å	41.3×10^{-6}					
2300	28.3	130×10^{-6}				
2400	20.7	220		82×10^{-6}	$1700-2800 \text{ Å}$	
2480	13.9	180			190×10^{-6}	
2537	12.9 (?)*	110	39.9×10^{-6}	52		
2650	13.1	80	21.7	58		
2805	14.9	180	36.2	104	$2400-2800 \text{ Å}$	
2950	81.6	870	60.0	175	880×10^{-6}	200×10^{-6}

* Data unsatisfactory

Wyckoff's values are those required to give a survival ratio of 50 per cent rather than our "natural" $1/e = 1/2.718 = 36.8$ per cent. The values given here are therefore Wyckoff's own values divided by 0.69 and are directly comparable with ours.

All other values are based on incident energy rather than on absorbed energy, and hence are not directly comparable. They are, however, recalculated for a survival ratio of 36.8 per cent.

to the above analysis. In all cases, the slope of the line (and hence the sensitivity, $1/\epsilon$) has been determined by the method of least squares, so that the judgment of the calculator does not enter into the final values.

Each point of an experiment such as is shown in Fig. 2 is the result of counts on more than a thousand bacteria sampled from the billions in the irradiated suspension. Each value of ϵ given in Table I is obtained from five to ten such observations, and the averages are the result of counts on fifty thousand or more organisms. Note how, for concentrations less than 10^9 bacteria per cubic centimeter, the value of ϵ decreases as the concentration increases, as is to be expected since the condition $\mu N_T = 1$ is more closely approximated for the larger values of N_T .

Table II shows how the sensitivity of the bacteria to ultraviolet radiation varies with the wave length. These values are the average of two or three experiments each. Values obtained by other investigators are included for comparison.

If our values of ϵ be plotted as a function of wave length, the curve shows a broad minimum at about 2650 Å, and although special efforts were made to detect a secondary maximum at about 2400 Å as described by Gates (11) no evidence of such a maximum could be found.

IV

DISCUSSION AND CONCLUSIONS

In the foregoing analysis, it has been assumed that the bacterial population possesses a uniform sensitivity. The grouping of the observed survival ratios about a semilogarithmic curve justifies this procedure to a first approximation. If, however, there is a distribution of sensitivities in the bacterial population, our survival ratio will be expressed by

$$N/N_0 = \int_0^{\infty} \phi(\epsilon) e^{-\frac{E}{N_T} \frac{1}{\epsilon}} d\epsilon$$

where $\phi(\epsilon)$ gives the distribution of the population as a function of ϵ . The direct solution of this integral equation to obtain a sensitivity distribution $\phi(\epsilon)$ from observed survival ratios is somewhat involved,

but is being carried through for a separate communication. Calculations made from several arbitrarily assumed sensitivity distributions showed that, unless the sensitivities have a very broad or very skew distribution, they will not affect the ϵ by more than about 6 per cent, which in general is within the experimental error for a given observation.

A number of factors which have not been considered, but which may have a secondary effect on the above energy calculations are (1) Double cells will have the effect of creating a secondary maximum in the sensitivity distribution, and will tend to increase the observed ϵ . An attempt has been made to consider this effect only in the case of young cultures where the fraction of double cells is large (70 per cent). (2) A portion of the incident ultraviolet is converted into fluorescent visible radiation (7, 13). It is unknown whether this energy is lost, or whether the fluorescent process or absorption of the fluorescent radiation are in themselves lethal processes. (3) The energy scattered back into the beam is lost, but this is probably only a very small percentage of the total. (4) The radiation scattered before it is finally absorbed is polarized to some extent. The work of Gates (11), however, indicates that polarization is not an important factor. (5) The non-viable (truly dead) organisms may not absorb energy at the same rate as the viable ones and those just inactivated by radiation. Such a consideration may, however, have a significant effect only in the case of old bacteria, where a large fraction (60–70 per cent) of the organisms are non-viable.

Of considerable interest is the difference in sensitivity between washed (6.13×10^{-6} ergs per bacterium) and unwashed (13.1×10^{-6} ergs per bacterium) standard bacteria. The smaller lethal dose for the washed material may be real because of a weakening of the cells, or it may be only apparent because of a removal of absorbing nutrient material and decomposition products from the surface of the organisms and from the suspending solution. The high variation in the energy required to inactivate young and old organisms (15.8 to 31.1×10^{-6} ergs per bacterium and 2.3 to 5.2×10^{-6} ergs per bacterium) is probably due to the difficulties of repeating experimental conditions, and too much importance should not be attached to the comparative results. Thus the high energy (22×10^{-6} ergs per bacterium) necessary to inactivate

the young ones may be due to the large number of double cells (70 per cent), while in the old cultures the large number of dead cells (60-70 per cent) may appreciably affect the calculated value of the sensitivity

The particular mechanism by which ultraviolet radiation inactivates bacterial cells is a question under considerable discussion. The effect has been attributed to the action of the radiation on the suspending material, on the cell wall, on enzymes in the organism, on the colloidal structure of the cytoplasm, and on nuclear material or material which controls cell division. Probably, however, the mode of action is very complex, involving several or all of these factors. Certainly, functions other than cell division are affected by "sublethal" doses of radiation, and if a number of them are involved in inactivation, it is obvious that no very simple explanation will suffice. One fact which stands out, however, is that the absorption curve and the sensitivity curve for *E. coli* both have a broad maximum at about 2650 Å, i.e., the wave lengths most highly absorbed (at least in the band 2400 Å to 3000 Å) are also the most efficient in producing lethal effects. The mere process of absorption is not equivalent to the process of killing, for then the killing would be proportional to energy absorbed for all wave lengths. Herein perhaps will lie our most significant clue to the mechanism of the action of ultraviolet radiation, when the problem is finally solved.

It is possible, of course, that a variation in the absorption of the dead bacteria for different wave lengths would serve to mask the maximum at 2400 Å as reported by Gates (11) but it is more probable that for these shorter wave lengths, the increasing absorption of organic impurities in the suspension would serve to mask the effect, if it exists. This would seem to constitute the weakest point of our method, but we believe that the more than satisfactory comparison of our results with those of other investigators using different methods thoroughly justifies the use of our liquid suspension method, and makes it available for types of experiments which cannot be performed when the bacteria must be plated on agar before irradiation.

The authors wish to thank Prof. B. M. Duggar for the advice and assistance he has given during the course of this investigation. The work was conducted under the auspices of the Committee on Radia-

tion of the Division of Biology and Agriculture, National Research Council

V

SUMMARY

1 The irradiation of bacteria in liquid suspension has been made possible through (a) the use of a specially balanced physiological salt solution which is practically non-absorbing for the wave lengths used, and which is of such composition that subsequent dilution of the bacterial suspension gives the proper number of organisms, (b) special design of the exposure cell and a very thorough method of stirring which subjects each organism equally to the radiation, (c) practically complete absorption of the incident radiation, through the use of very dense suspensions, thus eliminating the necessity for a separate determination of the absorption coefficients of the bacteria for the wave lengths used

2 The method also provides a means for determining the effects of sub-lethal doses

3 A formula is given for calculating from observed survival ratios the energy required to inactivate bacteria with ultraviolet radiation. The formula corrects for the protective action of non-viable organisms

4 Data are given for the inactivation of 15 hour and 240 hour cultures of *E. coli*, washed and unwashed, and for 6-7 hour cultures, unwashed. These data are compared with those of other investigators

5 A possible explanation for the differences in energy required to inactivate old, young, and standard cultures of bacteria is suggested

6 The possible mechanism of the action of ultraviolet radiation on microorganisms is discussed.

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THE SOLUBILITY OF *d* VALINE IN WATER*

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The present report is a continuation of the previously reported studies on the solubility of various amino acids in water (1). It is illustrative of the fact, which was not found in the case of the other compounds, that the mode of obtaining the crystals may influence the solubility. The technique used in the determination of solubilities was the same as that which has been described previously (1).

The *d* valine was a Hoffmann La Roche product which gave theoretical nitrogen and optical rotation values. The solubilities, which are given in Curve 1 of Fig. 1, were obtained by permitting a super saturated solution which was prepared by dissolving the product at temperatures higher than those given on the curve to cool at the indicated temperatures. A second product was prepared by the addition of alcohol to a concentrated solution of the amino acid. To obtain the points which lie on Curve 3, these crystals were dissolved in water at 25° and shaken in the presence of an excess of the solid phase. Curve 4 is a continuation of Curve 3. The points which lie on Curve 5 were obtained by dissolving the alcohol precipitated crystals in water in the zone between 0–16° and shaking the mixture in the presence of an excess of the crystals. On raising the temperature of this mixture beyond 31°, the points which lie on that portion of Curve 2 which joins Curves 4 and 5 were obtained. The points which lie on the balance of Curve 2 were obtained by raising the temperature of mixture 4.

The difference in crystal structure of *d* valine is shown in Figs. 2 and 3. Fig. 2 represents the crystals which correspond to Curve 1 of

* Aided by a grant from The Chemical Foundation Inc., and the Research Board of the University

Fig 1, and Fig 3 represents the crystals which were obtained by the addition of alcohol to the aqueous solution of the amino acid. These crystals correspond to Curve 3 or 5 of Fig 1, depending on the conditions under which they were dissolved and crystallized

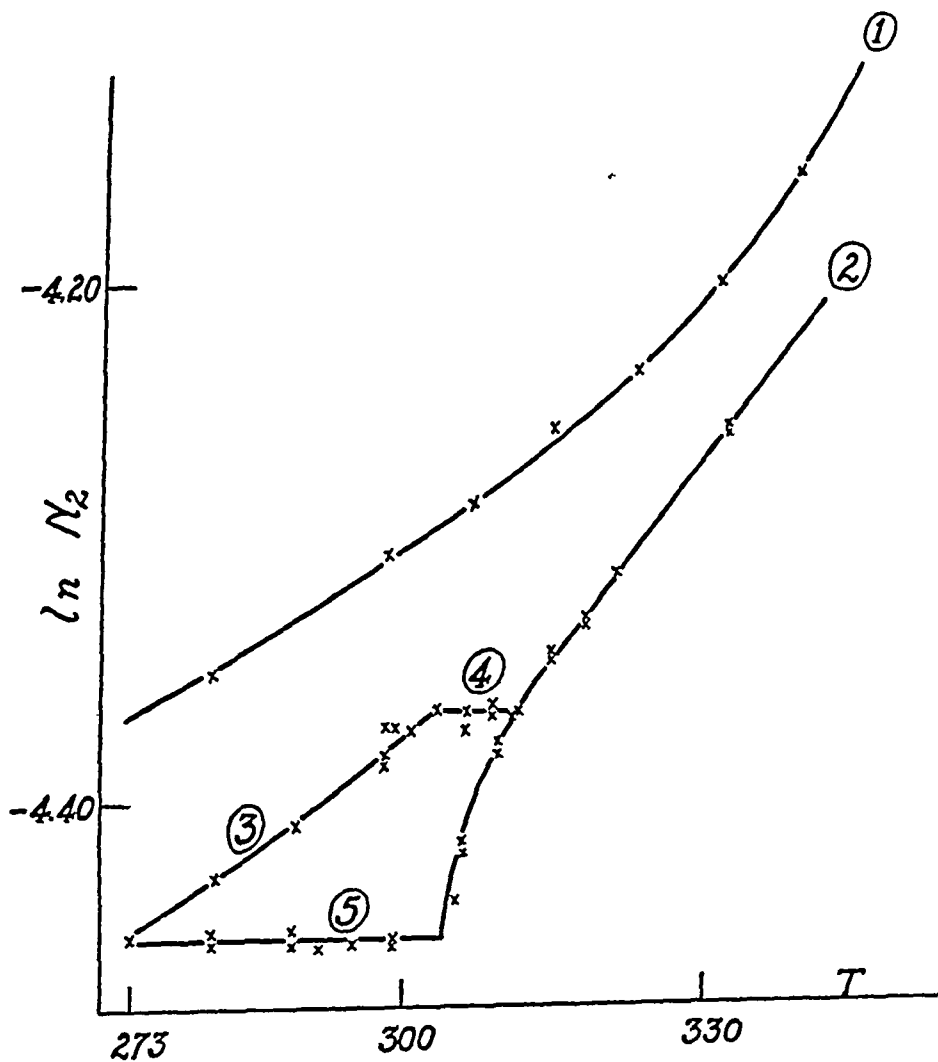


FIG 1 Solubility of *d*-valine in water

The data for the solubility equations of the curves which are graphically represented in Fig 1 are given in Table I. The solubility of *d*-valine at 25° (Curve 1) is in approximate agreement with the value



FIG 2

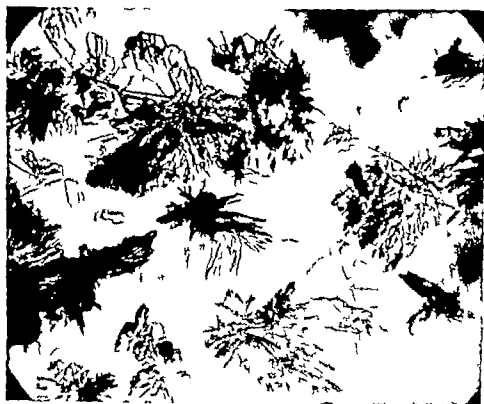


FIG 3

SOLUBILITY OF *d*-VALINETABLE I
Coefficients of Solubility Equations of the d-Valine Curves Given in Fig 1*

Curve No.	a_1	$b_1 \times 10^4$	$c_1 \times 10^6$	a	a_3	$b_3 \times 10^3$	$c_3 \times 10$	a_1	$b_1 \times 10^3$	$c_1 \times 10$	Maximum deviation †	Mean deviation †
											per cent	per cent
1	1 9211	8 1515	8 589	-0 1456	0 6274	-8 927	1 978	-3 4570	-8 528	1 9058	+0 91, +0 90	±0 41, ±0 37
2	1 6675	97 75	80 22	-0 4011	-20 8468	123 4	18 47	-24 2293	119 4	17 85	-0 95, -0 86	±0 62, ±0 69
3	1 8847	11 42	4 799	-0 1839	-0 3175	-3 406	1 105	-3 6570	-8 125	1 910	-0 24, +0 25	±0 14, ±0 15
†	1 9227			-0 1459	-0 3359			-4 3633			-0 66, -0 63	±0 28, ±0 26
5	1 8846			-0 1850	-0 4260			-4 4542			+0 66, +0 69	±0 31, ±0 34

* Solubility equations

$$\begin{aligned}\log S &= a_1 + b_1 t + c_1 t^2 \\ \log m &= a + b_1 t + c_1 t^2 \\ \ln m &= a_3 + b_3 T + c_3 T^2 \\ \ln \Lambda &= a_4 + b_4 T + c_4 T^2\end{aligned}$$

† The first figure given in this column refers to the first three of the above equations, the second to the last equation

found by Cohn and his coworkers (2) The value for ΔH of *d* valine at 25° is 500 calories The previously determined value for ΔH of *dl* valine is 1590 calories (3) The data indicate that *dl* valine is a racemic compound

It is of interest to note that over the ranges represented by Curves 4 and 5, the temperature is apparently without influence on the solubility of the *d* valine It is possible that these regions represent false equilibria, since the findings reported here are inexplicable from the standpoint of the classical concepts of the phase rule A true equilibrium might have been obtained had the mixtures been shaken for a sufficient length of time It would be expected also that, since the several crystal forms of *d* valine represent the same compound, the less soluble form must be the more stable and hence, on continued shaking, the transformation of the more soluble to the less soluble form should eventually take place From an experimental standpoint this is, however, not practicable, since, after several days, there is danger of bacterial decomposition

The findings that the solubility of a compound varies with the crystal form are not new Rabe (4) has shown that the solubility of thallos picrate depends on the particular crystal form of this compound

SUMMARY

1 The solubility of *d* valine in water has been determined over a range of 0–60°

2 The solubility of this amino acid varies with the mode of crystallization, indicating a dependence of solubility on the crystal form

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THE SOLUBILITIES OF THE *l*-DIHALOGENATED TYROSINES IN ETHANOL-WATER MIXTURES AND CERTAIN RELATED DATA*

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(Accepted for publication, September 24, 1935)

The solubility relationships of the amino acids in ethanol water mixtures are important both from the theoretical as well as from the practical standpoint. Decrease in the dielectric constant of the solvent, such as is produced by the addition of ethanol or acetone, is usually accompanied by a decrease in the solubilities of the amino acids (1). *l*-Tyrosine behaves in this respect like the majority of the aliphatic amino acids. However, in the case of the dihalogenated tyrosine compounds, it is found that their solubilities are markedly increased by the addition of ethanol to a certain concentration. Further addition of ethanol leads to a decrease in solubility. Evidently, factors other than the dielectric constant are concerned.

The importance of this fact is brought out in the preparation of diiodo tyrosine, according to the procedure of Oswald (2). He used ethanol for purposes of crystallization. Excess iodine is thus removed. Very poor yields of diiodo tyrosine are obtained. By following this procedure, it was found that the mother liquor, on cooling, would often become a transparent solid gel. This suggested that solvation, which usually increases solubility (3), had taken place. These observations led to the present investigation on the solubilities of *l* tyrosine and its dihalogenated substitution products in ethanol water mixtures.

The compounds used in the present investigation were prepared according to the procedures which have already been described (4). *dl* Thyroxine was a Hoffmann La Roche product. It was used without further purification. The technique employed in the determina-

* Aided by a grant from the Research Board of the University

tion of solubility and the quantitative estimation of the amino acids was the same as that which has been given elsewhere (4). The concentrations of *dl*-thyroxine in the saturated solutions were obtained by iodine determinations. The amounts of ethanol in the ethanol-water mixtures were found by density determinations. All solubility determinations in alcohol-water mixtures were carried out at 25°. Due to the viscosity of certain of the solvents used for the solubility estimations of *dl*-thyroxine, the solubility of this compound was determined at 30°.

Experimental Results

Twenty solubility determinations on *l*-tyrosine in 5 different ethanol-water mixtures were carried out. The data are represented graphically by Curve I in Fig. 1 in which the logarithm of the solubility ratio, $\frac{N_2}{\bar{N}_2^0}$, is plotted against the mol fraction, N_3 , of ethanol in the solvent.

The ratio, $\frac{N_2}{\bar{N}_2^0}$, refers the solubility of the amino acid in the ethanol-water mixture to its solubility in water. The solubility of *l*-tyrosine decreases as the concentration of ethanol is increased. Its solubility in ethanol ($N_2 \times 10^5 = 0.5$) is somewhat lower, but of the same order of magnitude as was found by Cohn and his coworkers (1) for a number of aliphatic amino acids. The ratio, $\frac{N_2}{\bar{N}_2^0}$, for tyrosine (1.0×10^{-1}) is larger than for the other amino acids which have been reported due to the low solubility of *l*-tyrosine in water.

Dichloro-*l*-tyrosine, when recrystallized from water, contains water of crystallization (4). Thirty-two solubility determinations of dichloro-*l*-tyrosine (hydrated) in eight concentrations of ethanol-water mixtures were carried out. The results are represented graphically by Curve III in Fig. 1. The solubility of this compound increases as the mol fraction of ethanol in the mixture is increased until a maximum is reached. At this point the mol fraction of dichloro-*l*-tyrosine in the ethanol-water mixture is about four times larger than the mol fraction in its saturated aqueous solution. On further increase of ethanol, the solubility of dichloro-*l*-tyrosine is decreased.

In determining the solubility of dichloro-*l*-tyrosine (hydrated) in

ethanol, it was found that the solubility, after shaking the mixture for 3 days, was greater than was to be expected in relation to the solubility found in other ethanol water mixtures. The solubility of this compound in ethanol with time is given in Table I. The excess of solute was found to have a different appearance after being in contact with

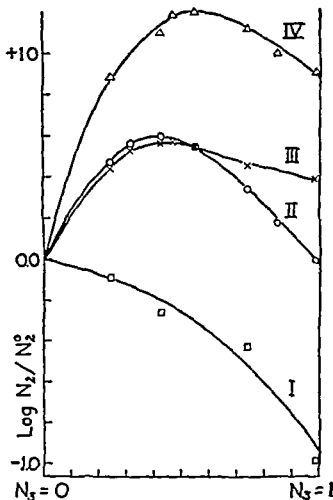


FIG 1 The relationship between the logarithm of the solubility ratio and the mol fraction of ethanol in the alcohol water mixture for *l*-tyrosine (Curve I), dibromo *l*-tyrosine (Curve II), dichloro *l*-tyrosine (Curve III) and diiodo *l*-tyrosine (Curve IV). Maximum error in data: *l*-tyrosine and diiodo *l*-tyrosine ± 5 per cent; dibromo- and dichloro *l*-tyrosine ± 1.6 per cent.

ethanol for 6 days than when it was in contact with ethanol for 3 days. Microscopically it had a different crystal form (Fig 2) than when crystallized from water (4). Recrystallization from hot ethanol gave the same crystal form as shown in Fig 2. Analysis of this form showed it to be free of water of crystallization. The high solubility found after shaking for several days was probably due

to the presence of both forms of dichloro-*l*-tyrosine in the mixture. The determined solubility during this time represented the solubilities of the two forms. After all of the hydrated form had been changed to the anhydrous form, the solubility became constant and represents the solubility of the latter form in ethanol (plus the small amount of water resulting from that which represented water of crystallization).

Dibromo-*l*-tyrosine crystallizes from water in two forms depending on the conditions under which crystallization is carried out (4). Thirty-two solubility measurements of dibromo-*l*-tyrosine (anhydrous) in eight concentrations of ethanol-water mixtures were carried out. The results are represented graphically by Curve II in Fig. 1. As in the case with dichloro-*l*-tyrosine, the solubility increases as the mol

TABLE I

Change of the Solubility of Dichloro-l-Tyrosine (Hydrated) in Ethanol at 25° with Time

Time	Solubility
<i>hrs</i>	<i>gm per 1000 gm solvent</i>
4	3 768
8	4 131
24	4 015
72	3 950
120	1 914
144	1 889
168	1 906

fraction of ethanol is increased until a maximum is reached. This occurred at approximately the same concentration of ethanol as with dichloro-*l*-tyrosine. The mol fraction of dibromo-*l*-tyrosine at its maximum solubility is about four times as great as that in a saturated aqueous solution. Further addition of ethanol to the mixture led to a decrease in solubility. The solubility of dibromo-*l*-tyrosine in ethanol is approximately the same as in water.

Thirty-two solubility determinations of diiodo-*l*-tyrosine in eight concentrations of ethanol-water mixtures were carried out. The results are represented graphically by Curve IV in Fig. 1. As with the two other dihalogenated tyrosines, the solubility of this compound increases as the mol fraction of ethanol in the mixture is increased until

a maximum is reached. The maximum occurs at a higher concentration of ethanol than in the case of the other dichlorogenated tyrosines. At the maximum point the mol fraction of diiodo *l*-tyrosine is about

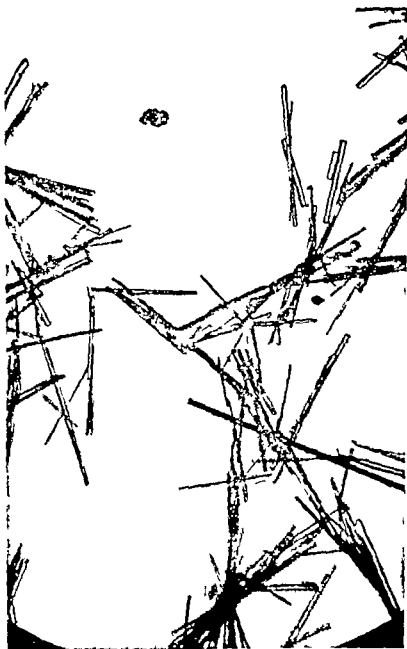


FIG. 2. Anhydrous form of dichloro *l*-tyrosine.

fifteen times as great as its solubility in water. The solubility in ethanol is considerably higher than in water. It was noted that the excess of solute in the higher ethanol-water mixtures changed in appearance from a crystalline substance to a transparent gel. It

resembled the material which is sometimes obtained when diodo-*l*-tyrosine is recrystallized from ethanol

The effect of temperature on the solubility of diodo-*l*-tyrosine in an ethanol-water mixture ($N_3 = 0.470$) was also determined. At this concentration of ethanol, the solubility of diodo-*l*-tyrosine is approximately at its maximum. Twelve solubility determinations were carried out at five different temperatures between 0° and 47.5° . The equation which expresses the solubility of diodo-*l*-tyrosine as a function of temperature was calculated to be

$$\ln N_2 = 4.01 \times 10^{-4} T - 19.808$$

On the assumption that the equation which was used to calculate the apparent differential heat of solution in water (4) can be applied in this

TABLE II
Solubility of dl-Thyroxine in Various Solvents at 30°

Solvent	Solubility
	<i>mg. per 1000 gm. solvent (± 10 per cent)</i>
40 per cent aqueous urea solution	12
Ethanol	14
Dioxan	17
Ethylene glycol	195
Propylene glycol	240

case also, the value for $\Delta H_{298^\circ} = 7090$ calories per mol at saturation. This value is somewhat less but of the same order of magnitude as that found by Dalton and Schmidt (5) in the case of a saturated aqueous solution of diodo-*l*-tyrosine (7830 calories).

Attempts were made to determine the solubility of diodo-*l*-tyrosine in propylene glycol. The amino acid is unstable in this solvent. The solution begins to turn brown within 24 hours. Approximate solubility values at this time were twenty to twenty-five times larger than the solubility value of diodo-*l*-tyrosine in water.

A few solubility determinations were carried out on *dl*-thyroxine. On the basis of iodine estimations, no measurable solubility in water was found. At 25° , the solubility in an ethanol-water mixture con-

taining 0.470 mol fraction of ethanol was 0.015 gm per 1000 gm of solvent. The solubility values of *dl*-tyrosine in various other solvents are given in Table II. Of those which were tried, propylene glycol was found to be the best solvent.

DISCUSSION

Cohn and his coworkers (1) have summarized the factors which affect the solubility of aliphatic amino acids in ethanol-water mixtures. When the volume fraction of ethanol is small, the logarithm of the solubility depends chiefly on the dielectric constant. The solubility relations of the longer hydrocarbon chain amino acids are more complex especially when the system contains large volume fractions of ethanol. In a general way, the solubility relations of *l*-tyrosine resemble those of the long hydrocarbon aliphatic amino acids.

The introduction of halogens into the hydroxyphenyl ring affects the solubility of the resulting compounds in ethanol-water mixtures to such a degree that the factors other than the dielectric constant appear to play important rôles. The present data are not sufficient to warrant making precise conclusions. However, the nature of the solvent as well as the specific chemical groups in the molecule are factors which affect solubility. In the case of the dihalogenated tyrosine compounds at least two important factors operate to affect their solubility. One is the hydrocarbon chain. The effect of the addition of ethanol is to decrease its solubility. The presence of the hydroxyphenyl ring in the molecule is without decided influence. The effect of the presence of the halogens in the ring, probably as a result of orientation to the ethanol molecules, is to increase the solubility of the dihalogenated tyrosine compounds. This effect is opposite to that of the hydrocarbon chain. The halogen effect predominates over the effect of the latter up to a certain concentration of alcohol. With further increase in ethanol concentration, the effect of the hydrocarbon chain becomes more pronounced, with the result that the solubility decreases. The effect of the halogens continues to be considerable, however, with the result that the solubility of the dihalogenated tyrosine compounds in alcohol is decidedly greater than that of tyrosine.

SUMMARY

1 The solubilities of *l*-tyrosine, dichloro-*l*-tyrosine (hydrated), dibromo-*l*-tyrosine (anhydrous), and diiodo-*l*-tyrosine in ethanol-water mixtures at 25° have been determined

2 It was found that the solubilities of the dihalogenated substitution products of *l*-tyrosine are increased by addition of ethanol up to a certain concentration. Further addition of ethanol leads to a decrease in solubility. The solubility of *l*-tyrosine is decreased by addition of ethanol.

3 Dichloro-*l*-tyrosine (hydrated) was found to change to the anhydrous form when allowed to stand in the presence of ethanol.

4 The apparent heat of solution of diiodo-*l*-tyrosine in an ethanol-water mixture has been determined.

5 The solubility of *dl*-thyroxine at 30° has been determined in urea solution, ethanol, dioxan, ethylene glycol, and propylene glycol.

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PIGMENTS OF THE RETINA

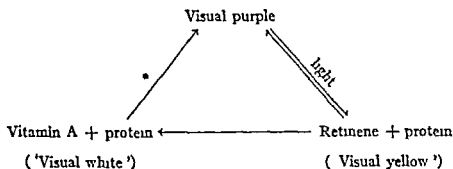
I THE BULL FROG

By GEORGE WALD

(From the Biological Laboratories, Harvard University, Cambridge)

(Accepted for publication September 25 1935)

The participation of certain carotenoids in the visual purple system of frogs is partly expressed in the equations (Wald, 1935-36)



All of these reactions but the one starred occur in the isolated retina as well as in the intact eye

In the present paper the components of this system in the bull frog, *Rana catesbiana*, are analyzed in a series of simple experiments, the results of which are presented in objective, and I believe unequivocal form

Observations are also reported upon the distribution and properties of vitamin A, xanthophyll, and flavine in the pigmented layers of the eye

Retinas

The retina contains varying amounts of the carotenoids vitamin A and retinene. Dissolved in chloroform in the concentrations here considered, vitamin A is colorless, retinene greenish yellow. The color of retinal extracts under various conditions thus offers a first indication of changes in these substances. With antimony trichloride reagent, both carotenoids yield blue colorations, due in the case of

vitamin A to an absorption band at 612–615 $m\mu$ (crude extracts), in that of retinene to one at 662–666 $m\mu$. This reaction is used to identify both substances in the following experiments

Dark Adapted Retinas Visual Purple and Bound Retinene—Dark adapted retinas may be extracted thoroughly in the dark with benzine or carbon disulfide without injury to the visual purple. The extracts

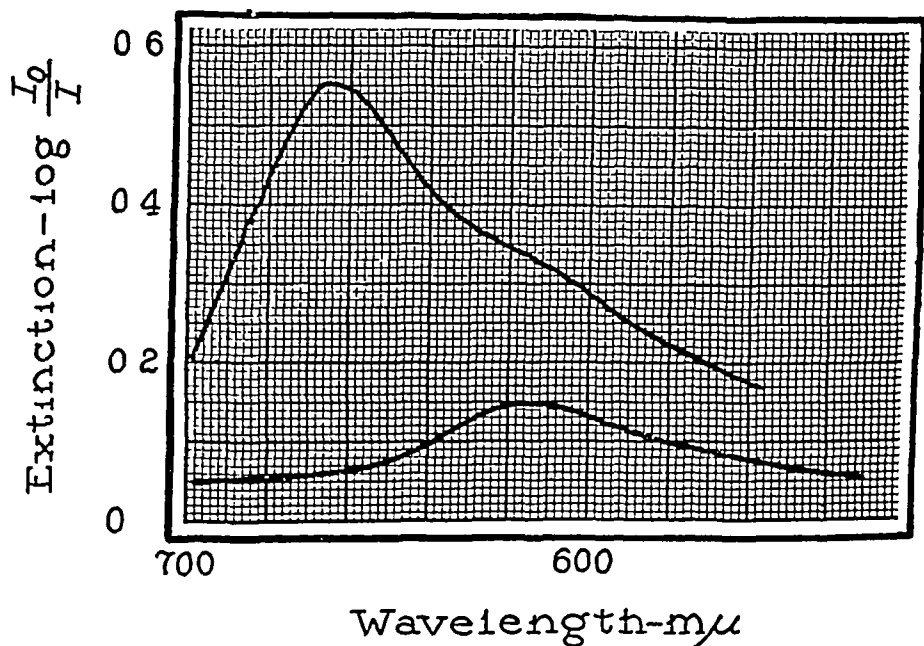


FIG 1 The liberation of retinene from visual purple by chloroform. Spectra of antimony trichloride reactions with a benzine extract of dark adapted retinas (lower curve), and with a subsequent chloroform extract of the same retinas (upper curve). Benzine withdraws only a trace of vitamin A (615 $m\mu$ chromogen). Chloroform destroys the visual purple and extracts a large quantity of retinene (665 $m\mu$ chromogen). If the ordinates of the upper curve are multiplied by about 4, the heights of the curves represent approximate relative concentrations.

are colorless and contain a very small quantity of vitamin A alone. Subsequently, the same retinas may be extracted in the dark with chloroform, which almost immediately destroys visual purple. The chloroform extract is greenish yellow, and contains a large quantity of retinene.

Retinene is freely soluble in benzine or carbon disulfide after it has been extracted from the retina, it must be bound in the dark.

adapted tissue to some material insoluble in these reagents. Chloroform liberates it simultaneously with the destruction of visual purple.

These relations are shown in Fig 1, which presents the results of the following experiment¹

Experiment—The retinas of four dark adapted frogs were shaken thoroughly in the dark with a total of about 6 cc benzene in three successive portions. The retinas were next extracted similarly in the dark with chloroform. The combined benzene extracts were brought into 0.3 cc. chloroform and the colorless solution used in a single antimony trichloride test. The result is shown in the lower curve of Fig 1. The chloroform extract was greenish yellow. Tested with antimony trichloride, it yielded the upper curve of Fig 1. Since only about $\frac{1}{4}$ the chloroform extract was used in the latter test, the ordinates of the upper curve should be multiplied by about 4 to make the heights of the curves comparable.

The Liberation of Retinene by Light—The initial extraction of dark adapted retinas in the dark with benzene may be followed by re-extraction with benzene in bright light. Light bleaches the visual purple to a bright orange color (visual yellow), after which benzene readily extracts the total retinene. The tissue residue is colorless.

Retinene is therefore liberated by light—as by chloroform in the preceding experiment—in the destruction of visual purple. Fig 2, obtained in the following experiment, illustrates this relation.

Experiment—Four dark adapted retinas were shaken violently in the dark in a shaking machine for 15 minutes with a total of 6 cc benzene in 3 successive portions. Precisely this process was repeated with the same retinas in bright day light. Each extract was brought into 0.3 cc chloroform. The 'dark' one was colorless, the 'light' one bright yellow. Both were tested with antimony trichloride. The former yielded the lower curve of Fig 2, the latter the upper one.

Conversion of Retinene to Vitamin A in the Isolated Retina—The retinene liberated by light is converted quantitatively to vitamin A in the isolated retina. This process is complete in about an hour at

¹ The spectra shown in this paper were measured with a recording photoelectric spectrophotometer designed by Professor A. C. Hardy at the Massachusetts Institute of Technology (Hardy 1935). The curves were drawn on coordinate paper by the instrument itself and have been merely mounted and reproduced. Absorptions are plotted as extinction or optical density, $\log(I_0/I)$ in which I_0 is the incident and I the transmitted intensity. This quantity is directly proportional to concentration and to the depth of the absorbing layer.

25°C It is evidenced by the fading of the bleached (visual yellow) retina to colorlessness Fig 3, the result of the following experiment, demonstrates this change

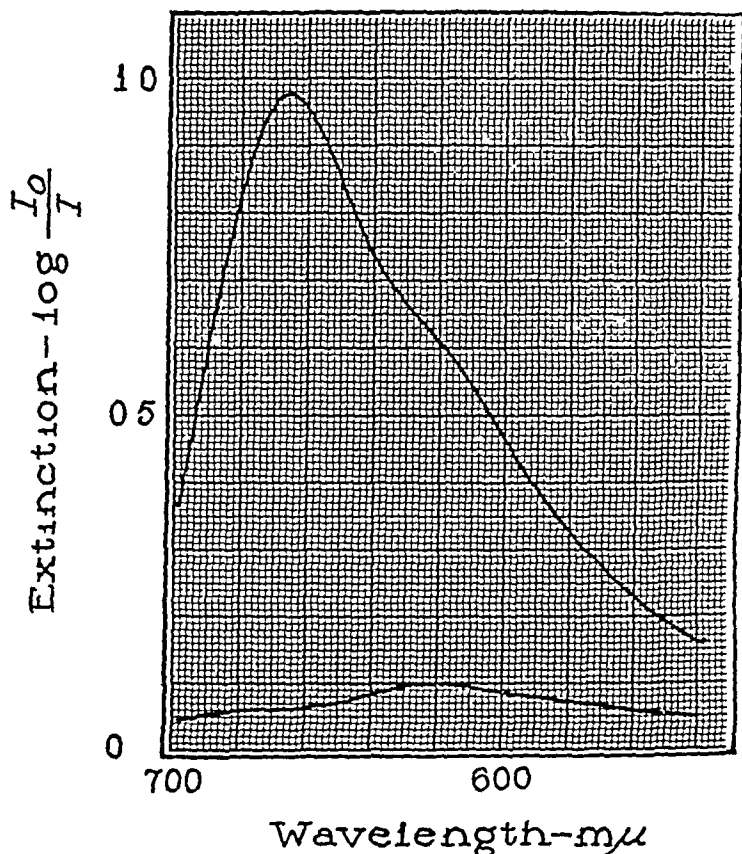


FIG 2 The liberation of retinene from visual purple by light Spectra of antimony trichloride reactions with benzine extracts of dark adapted retinas (lower curve),² and of the same retinas subsequently bleached to the visual yellow stage in bright light (upper curve) Compare with Fig 1

Experiment—Right and left retinas from eight dark adapted frogs were separately prepared One set of eight retinas was extracted in the dark with about 12 cc chloroform in four portions The extract was concentrated to about 1.5 cc A sample of this, tested with antimony trichloride, yielded the upper series of curves in Fig 3 The curves were measured consecutively on a single antimony trichloride test and follow the fading of the blue color produced in this reaction

² This curve was drawn 5 mμ too high in wavelength due to a fault in calibration which was corrected before the upper curve was recorded

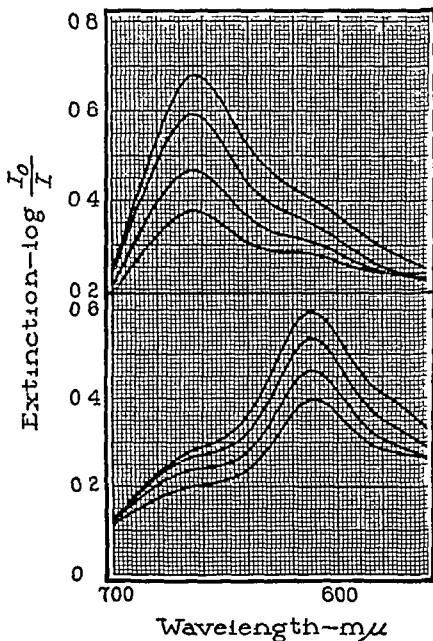


FIG 3 The conversion of retinene to vitamin A Spectra of antimony trichloride reactions with chloroform extracts of dark adapted retinas (upper series) and of retinas from the same animals bleached and allowed to fade to colorlessness (lower series) Retinene (665 mμ chromogen), bound in the dark adapted retina is liberated by light and converted during the fading process to vitamin A (612 mμ chromogen) Each series of spectra consists of successive measurements of a single antimony trichloride test, and follows for about 15 minutes the disappearance of the blue color produced in this reaction

The second set of eight retinas from the same frogs was bleached in bright day light and left in moderate light at 22 C for about an hour The retinas were then treated exactly like the former group In the antimony trichloride test they yielded the lower series of curves of Fig 3

Thermal Nature of the Conversion of Retinene to Vitamin A—The formation of vitamin A from retinene is a typical thermal reaction. It is inhibited enormously even in bright sunlight by cooling the retinas to 0°C. At room temperature it occurs in the dark with about the same speed as in the light. Fig 4, obtained in the following experiment, illustrates the latter phenomenon.

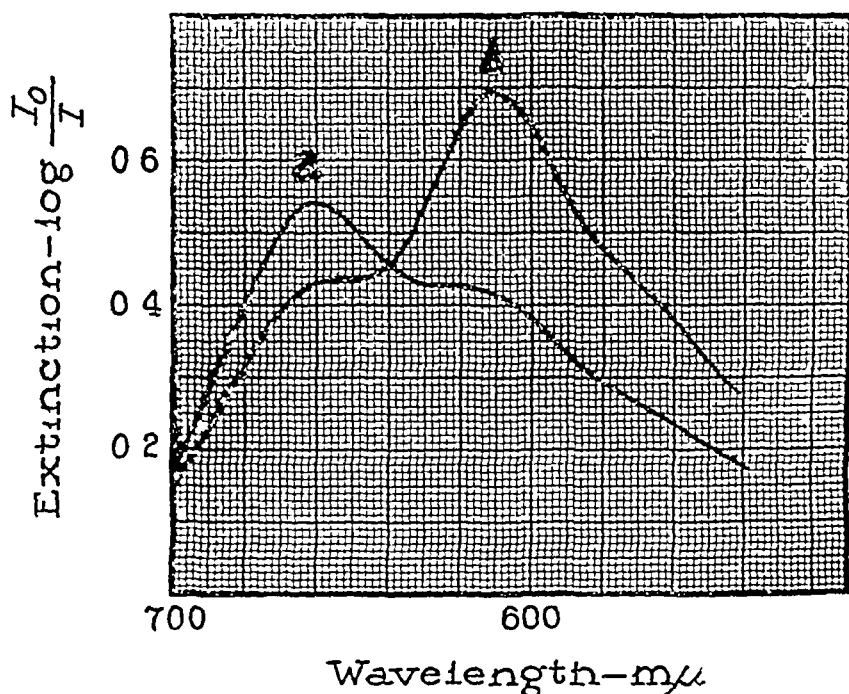


FIG 4 Conversion of retinene to vitamin A in the dark. Spectra of anti-mony trichloride reactions with extracts of retinas bleached to the visual yellow condition and replaced in darkness (a) extracted after 2 minutes in the dark, (b) extracted after 69 minutes in the dark. Compare with Fig 3.

Garten (1906) suggested that light decomposes visual purple directly into an equimolecular mixture of visual white and visual yellow, the latter reverting quantitatively to visual purple in the dark. This view is confuted by the experiment, which shows that visual white (*i e*, vitamin A and other colorless substances) is formed from visual yellow by a secondary process independent of the illumination.

Experiment—Right and left retinas of six dark adapted frogs were prepared separately. Both sets were simultaneously exposed to sunlight for 20 seconds, the visual purple was bleached almost instantly to a bright orange color. Both

sets were then placed in complete darkness at 22 C. After 2 minutes one set was extracted in the dark with chloroform. This extract yielded Curve *a* of Fig. 4. After 69 minutes in the dark, the second set of retinas was similarly extracted. It yielded Curve *b* of Fig. 4.

Light Adapted Retinas—In the isolated retina vitamin A is the final product of the bleaching and fading reactions. In the living animal, however, the vitamin is re synthesized to visual purple, completing

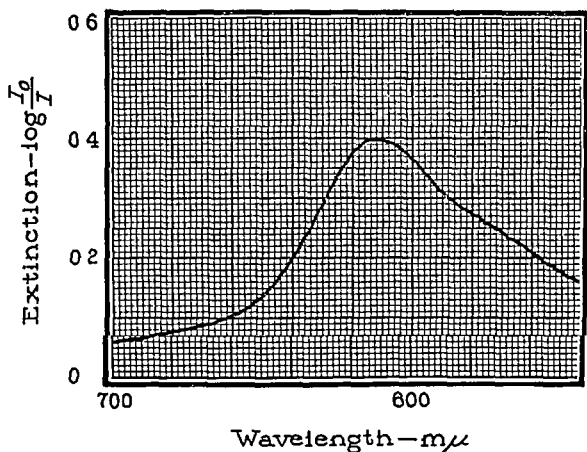


FIG. 5. Light adapted retinas. Spectrum of the antimony trichloride reaction with an extract of retinas from animals adapted to bright daylight.

the visual cycle. In the frog this process occurs even in extirpated eyes in which the relation of the retina to the pigment epithelium has not been disturbed (Ewald and Kuhne, 1878).

In a frog exposed to a constant intensity of illumination the visual cycle attains a steady state, in which the concentrations of visual purple, retinene, and vitamin A remain constant, each being formed as fast as removed. At low illuminations the steady state is close to the dark adapted condition, characterized by much visual purple and

little free vitamin A and retinene. In bright light it is displaced toward the opposite end of the cycle, resulting in an accumulation of vitamin A.

In accord with this analysis the retinas of frogs adapted to bright daylight are found to be colorless, and contain vitamin A alone, in quantities very much greater than dark adapted retinas. Fig 5 presents the result of the following typical experiment.

Experiment—Four frogs were exposed to bright diffuse daylight for $\frac{3}{4}$ –1 $\frac{1}{4}$ hours. The retinas, which were colorless, were prepared in daylight. Adhering pieces of pigment epithelium were carefully picked away with fine forceps. The cleared retinas were extracted with chloroform, and the concentrated extract tested with antimony trichloride. Fig 5 shows the result.

Concentrations Summary—Concentrations of vitamin A and retinene were measured by methods which have already been described (Wald, 1935–36). The amounts of these substances per retina found in groups of twenty retinas are included in the following table, which summarizes the results of the foregoing procedures.

Retinal condition	Pigmentation	Benzene extract	Chloroform extract
Dark adapted	Visual purple	Trace of vitamin A	Trace of vitamin A + 1.8 units retinene ³
Bleached	Visual yellow	Trace of vitamin A + 1.8 units retinene	
Bleached and wholly faded	Colorless (visual white)	2.3 γ vitamin A	
Light adapted	Colorless	0.8 γ vitamin A	

As in other frog species, isolated retinas which have been bleached and allowed to fade completely contain very much more vitamin A than retinas light adapted *in vivo*. I originally interpreted this observation to indicate destruction of vitamin A in the active retina, and assumed that it accounts for the dependence of the visual purple system in some animals upon a continuous accession of vitamin A in the diet (Wald, 1935–36).

This datum may be interpreted more simply. It is sufficient to

³ A unit of retinene is defined arbitrarily as ten times its optical density in chloroform solution at 430 $m\mu$ (Filter S43 of the Pulfrich photometer) in a layer 1 cm. in depth.

assume that in the intact eye some of the vitamin A liberated in light adaptation diffuses out of the retina into neighboring tissues. In the isolated retina, of course, this is impossible. Presumably, partition factors govern the distribution of vitamin A among the tissues. The small quantity of free vitamin found in the dark adapted retina is probably its equilibrium concentration. During light adaptation this is exceeded and some vitamin diffuses away. During dark adaptation the retina recaptures vitamin A by binding it in non diffusible form in visual purple.

It is unnecessary therefore to assume that vitamin A is destroyed in the visual cycle. The term, "degradation products," is superfluous in the diagram of the visual purple system (Wald, 1935-36). The organism's continuous demand for vitamin A must be ascribed to a loss in processes still unidentified.

Pigmented Layers

The combined pigment epithelium and choroid layer of an eye of *R. calesbiana* contain about 2% of xanthophyll and about 9% of vitamin A. At least 80 per cent of these quantities is located in the pigment epithelium alone. This single layer of cells, which comprises only about $\frac{1}{3}$ the total pigmented tissue and, which when dried weighs about 1.2 mg, therefore contains about 1.3 mg of xanthophyll and about 6.0 mg of vitamin A per gram dry weight.

Experiment—Four frogs in ice water were adapted to diffuse daylight and their retinas were prepared in a room kept at 6°C. Under these conditions most of the pigment epithelium attaches very firmly to the retina and is removed from the fundus with it leaving the choroid layer alone behind. The latter was scooped into Ringer's solution with a small spatula. The retinas were warmed to about 22°C. At this temperature the pigment epithelia adhere less closely and may be picked away from the retinas with forceps. Pigment epithelia and choroid layers were collected separately out of the Ringer in which they had been prepared by centrifuging. Each group of tissues was extracted with benzene and the concentrations of xanthophyll and vitamin A were measured.

	Combined pig- mented layers %	Per cent in pig- ment epithelium	Per cent in choroid
Xanthophyll per eye	2.2	77	23
Vitamin A per eye	6.9	84	16

Xanthophyll—Xanthophyll may be extracted from the pigmented layers with benzine. When the resulting solution is shaken with 90 per cent methanol, the pigment enters the benzine layer almost quantitatively (epiphasic). After saponification this partition is reversed, the pigment entering the methanol (hypophasic). Before saponification the pigment is strongly adsorbed from benzine by powdered aluminum oxide, though it passes readily through calcium carbonate. After saponification it is strongly adsorbed by the latter material.

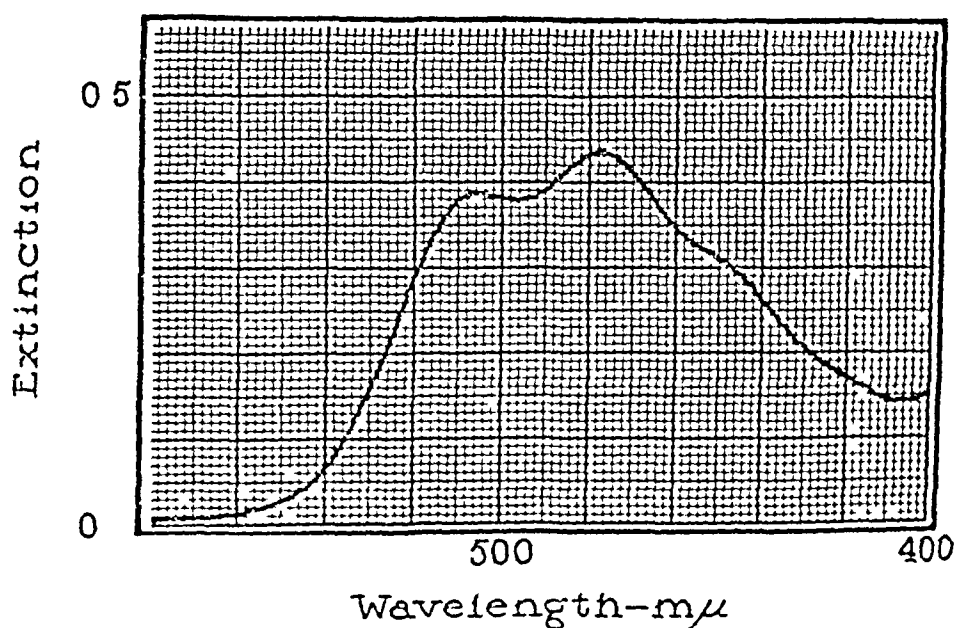


FIG. 6. Spectrum of a carbon disulfide solution of xanthophyll from pigmented tissues. This preparation was partly purified by saponification, partition between 90 per cent methanol and benzine, and adsorption on calcium carbonate.

These changes in behavior on saponification indicate that the xanthophyll occurs in the tissues as an ester.

Xanthophyll preparations from the pigmented layers deteriorate easily with age, yielding derivatives which may obscure the outcome of the experiments greatly. Portions of the pigment may turn permanently epiphasic though changing but little in spectrum. Other portions which remain hypophasic may shift several mμ in spectrum toward shorter wavelengths. They are adsorbed in a (CaO) column.

above the xanthophyll itself Kuhn and Brockmann (1932) have reported similar changes in xanthophyll preparations from plant tissues It was due to the presence of such impurities that the spectrum of a crude pigment layer preparation presented earlier diverged appreciably from that of crystalline xanthophyll (Wald, 1935-36)

The spectrum of free xanthophyll from pigmented tissue, purified by partition and adsorption on calcium carbonate, is shown in Fig 6 This agrees within the errors of measurement with the spectrum of crystalline xanthophyll (lutein, $C_{40}H_{54}(OH)_2$) (Kuhn and Smakula, 1931) The spectrum shown here is somewhat more diffuse than that of the crystalline material, due partly to the fact that it was measured with an instrument of wide slit-width ($5\text{ m}\mu$ at the objective) which tended to level maxima and minima slightly

These xanthophyll preparations, freed of vitamin A by adsorption on calcium carbonate, and concentrated in chloroform, yield a blue green color when mixed with antimony trichloride reagent, due to an absorption band at about $585\text{ m}\mu$ This response also is characteristic of crystalline xanthophyll (von Euler, Karrer, Klusmann, and Morf, 1932)

The methods for treating and identifying carotenoid extracts referred to here have been described by Kuhn and Brockmann (1932) and Karrer and Schöpp (1932), and have recently been reviewed in detail by Zechmeister (1934)

Vitamin A —When a saponified extract of pigmented layers in benzene is poured through a column of powdered CaCO_3 , xanthophyll is quantitatively adsorbed and vitamin A emerges alone in the colorless washings Brought into chloroform and treated with antimony trichloride reagent, it yields the sharp band at $620\text{ m}\mu$ specific for this vitamin (Fig 7) Crude extracts yield bands in the same reaction at 612 – $615\text{ m}\mu$ The shift to lower wavelengths is due apparently to the presence of contaminating substances, for the adsorption treatment alone moves the band to $620\text{ m}\mu$, even in unsaponified preparations⁴

Like the xanthophyll, vitamin A, if partitioned between 90 per cent

⁴ Fish liver oils show similar and more extreme behavior The antimony trichloride vitamin A band in crude oils is at 600 – $610\text{ m}\mu$ After partial purification this is shifted to $620\text{ m}\mu$ (Heilbron, Gillam, and Morton, 1931)

methanol and benzene, is epiphasic as extracted, but hypophasic after saponification. It appears therefore to occur in the tissues as an ester (compare Karrer, Morf, and Schopp, 1931). One preparation, partially purified by adsorption, exhibited the following properties

	Per cent in benzene	Per cent in methanol
Unsaponified	98.6	1.4
Partially saponified in 5 per cent ethanol KOH for 25 minutes at 40°C	59.9	40.1

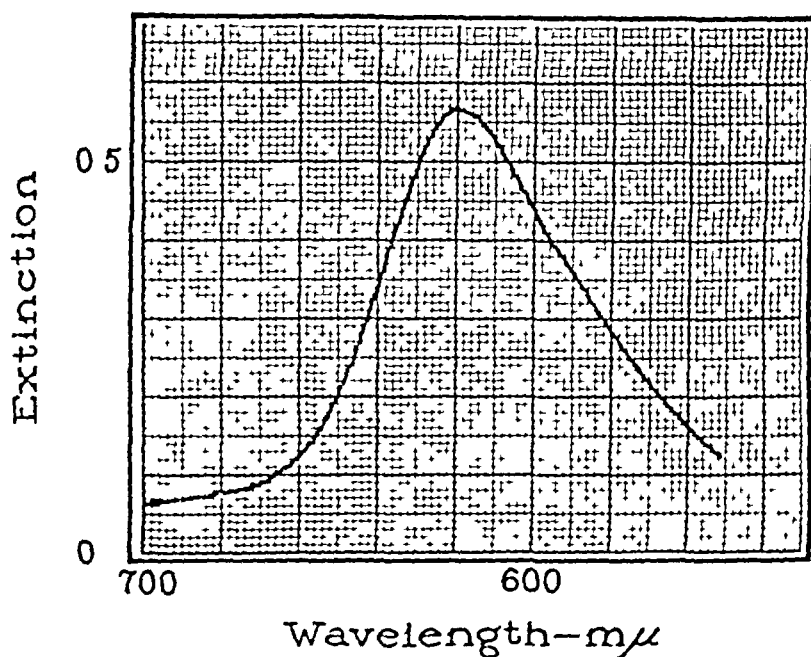


FIG. 7. Spectrum of the antimony trichloride reaction with vitamin A from pigmented tissues, freed from xanthophyll and other impurities by adsorption methods.

Fluorescence.—After pigmented tissue has been extracted exhaustively with benzene it may be re-extracted with 80 per cent aqueous acetone. The extract contains a lemon-yellow pigment which fluoresces strongly green. On adding acids or alkalis the fluorescence vanishes and returns on neutralizing. If a drop of dilute sodium hydro-sulfite solution is added to the extract made just alkaline to litmus the pigment is decolorized. On shaking with air it regains its yellow color. This

reaction may be repeated indefinitely. The properties identify the pigment as a flavine (lyochrome) (Kuhn, György, and Wagner Jauregg, 1933 *a, b*). Von Euler and Adler (1934 *a, b*) have reported the presence of flavines in the eye tissues of a number of fishes and mammals.

The absorption spectrum of crude flavine from bull frog pigmented layers is shown in Fig. 8. This agrees fairly well with the spectrum of similar extracts from fish pigmented layers (von Euler and Adler, 1934*a*) and with that of crystalline lactoflavine (Kuhn, György, and Wagner Jauregg, 1933 *b*). An incomplete extract of pigmented tissues from 28 eyes contained about 1.3 γ of flavine per eye. The measurement was

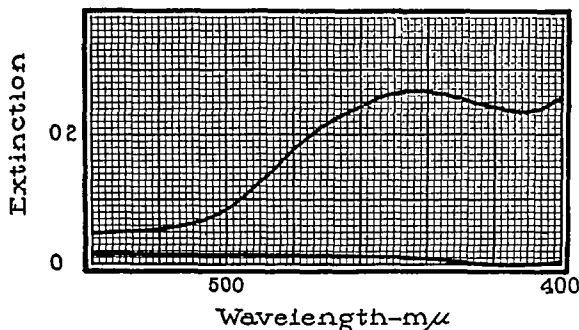


FIG. 8. Spectrum of a water solution of crude flavine from pigmented tissues. The lower curve is the reference spectrum of distilled water alone.

performed in the Pulfrich photometer, and is based on the fact that in this instrument 100 γ of crystalline lactoflavine per cubic centimeter of aqueous solution, measured with the S47 filter in a layer 1 cm. in depth, has an optical density of 3.2 ± 0.15 (Kuhn, Reinemund, Weygand, and Strobele, 1935).

Lactoflavine, obtained from whey, liver, eggs, and grass has been shown to be identical with vitamin B₂ or G (Kuhn, Rudy, and Wagner Jauregg, 1933; von Euler, Karrer, Adler, and Malmberg, 1934). Flavines combined with protein appear to form an extensive group of closely related redox enzymes, of which the *gelbe Ferment* of War

burg and Christian (1932) alone has been isolated (Theorell, 1935). No attempt has been made in the present work to determine whether the retinal flavine occurs in free or bound form in the tissues. In the fish eye von Euler and Adler found it to be almost entirely free, and restricted to the pigment epithelium. Its function in the eye is unknown. Von Euler and Adler have suggested that it may behave as a photosensitizer in the retina. It might well play some part in the extraordinarily powerful respiratory and fermentative system of this tissue.

Blue-Fluorescent Substances—Von Euler and Adler (1934 *b*) have described certain unidentified substances in fish pigmented tissues which possess very strong blue fluorescence. Water extracts of bull frog pigmented layers contain similar substances. No attempt has been made in the present work to identify them or measure their quantities.

SUMMARY

1 The interrelations of visual purple, retinene, and vitamin A in the bull frog retina are analyzed in simple experiments, the results of which are presented in a series of automatically recorded spectra.

2 Observations are reported upon the distributions, properties, and concentrations of xanthophyll, vitamin A, and flavine in the pigmented tissues of the eye.

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THE DOUBLE REFRACTION OF CHITIN

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I

Chitin, a nitrogen containing polysaccharide, is the chief structural element in the cell walls of fungi and in the integument of arthropods. Structurally chitin resembles cellulose, the unit of structure being the acetyl glucosamine residue instead of the glucose residue (Meyer and Mark, 1930). Organized chitin is optically anisotropic and exhibits birefringence which is ordinarily positive¹ in relation to the principal axis of the structure in which it occurs. Attempts to analyze the double refraction of chitin by means of the imbibition technique of Ambronn (Ambronn and Frey, 1926) have ascribed to it two components: (1) a positive double refraction due to the parallel arrangement of minute, submicroscopic micelles² of chitin imbedded in another medium (*form birefringence*), and (2) a negative double refraction of the crystalline micelles themselves. The latter, if correct, is remarkable since the micelle of cellulose is strongly optically positive.

The theory of the imbibition technique as developed by Ambronn may be briefly stated as follows (see Ambronn and Frey, 1926, Schmidt, 1934, Frey Wyssling, 1935). Bodies exhibiting form birefringence are two phase systems, consisting usually of a solid framework permeated by a continuous medium such as gas or liquid. If the dimensions of the solid units and the spaces between them are small in

¹ Birefringence is referred to as positive in relation to some axis when the greatest index of refraction of the substance n_γ , is parallel to, negative when n_γ is perpendicular to that axis.

² Micelle is here used in the sense of Naegeli to denote an aggregate of submicroscopic dimensions of smaller chemical units. Polarization optics can assign no definite limits of size to the micelle, but can only tell us that it is an elongate structure. For cellulose a recent estimate of the micellar dimensions is $60 \times 60 \times 750 \text{ \AA}$ (Frey Wyssling 1935).

crayfish's carapace A variety of evidence points to the chemical similarity of chitin from plant and animal sources (Rammelberg, 1931, Khouvine, 1932, Zechmeister and Toth, 1934) All the measurements described below were made on crayfish chitin

A small piece of the edge of the crayfish's carapace was cut off, with marginal hairs attached These hairs are attached to and continuous with the chitinous inner layer of the shell which, in the case of the lobster, was used by Möhring (1926) as a source of chitin After boiling in saturated KOH following the method of Campbell (1929), the hairs acquire a violet color with iodine in KI plus dilute H_2SO_4 dissolve in 70 per cent H_2SO_4 , and are therefore judged to be chitin Before imbibition, fresh hairs were boiled briefly in 5 per cent HCl to remove any calcareous incrustations, and then in 10 per cent KOH to dissolve any other materials in the hair which might impede penetration This procedure was found to facilitate imbibition greatly without sensibly altering the double refraction

Retardations were measured by means of a Berek compensator used in a Fuess polarizing microscope Blue light of high intensity was used throughout obtained by focusing the beam of a 250 watt projection lamp on the substage mirror and screening out heat and other portions of the spectrum with a 2 cm filter of saturated aqueous $CuSO_4$ Wavelength 486 m μ was used in all the computations

Imbibition was carried out in hollow ground glass slides containing about 0.2 cc. of fluid and closed by a large cover glass A small air bubble was enclosed, which, when the slide was tilted back and forth, served to give excellent mixing of the fluid in the chamber Being sealed by capillary force between the slide and cover glass, the chamber could be heated to 60°C without serious evaporation of one constituent of a mixture With heating imbibition occurred very rapidly usually no further change being noted after 5 minutes In doubtful cases an imbibition time of several days was allowed

The refractive indices of all liquids and mixtures below $n = 1.60$ were measured with a Pulfrich refractometer, and the values are given below as n_D at room temperature (approximately 25°C) Higher values of n were obtained by extrapolation of mixture curves measured over the working range of the instrument All retardation values are calculated from averages of at least three pairs of settings of the compensator

Three principal series of imbibition fluids were used (1) water mercuric KI mixtures³ (2) xylol methylene iodide mixtures and (3) ethyl alcohol (or xylol) iodobenzene mixtures The results are given in Figs. 1, 2, and 3 In addition,

³ A saturated solution of mercuric potassium iodide (potassium iodomercurate Thoulet solution) was prepared by adding KI and HgI_2 in excess to water at room temperature This saturated solution has a refractive index of approximately 1.73

a short series was run with water-glycerol mixtures (Fig 4) All of these measurements were made at one particular spot on the same crayfish hair within a period of 2 weeks As may be seen from duplicate measurements, the curves were reversible and reproducible

Sources of error other than instrumental in this kind of work are chiefly due to (1) incomplete penetration of the fluids, (2) swelling or shrinkage of the material with possible alteration of the shape and relative volumes of the micelles and the spaces between them, (3) chemical effects of the fluids on the imbibing material With crayfish chitin penetration was taken to be complete when further heating and rinsing in fresh fluid produced no additional change in birefringence Measurable swelling did not occur even in the mercuric KI solutions The possible occurrence of unforeseen chemical or adsorption effects is a real one Cinnamic aldehyde, benzaldehyde, and to some extent aniline were found to reverse the normal positive double refraction of chitin, producing negative double refraction This type of action was studied with collagen fibers by von Ebner (1894), and attributed by him to an oriented adsorption of imbibed molecules on the colloidal framework

III

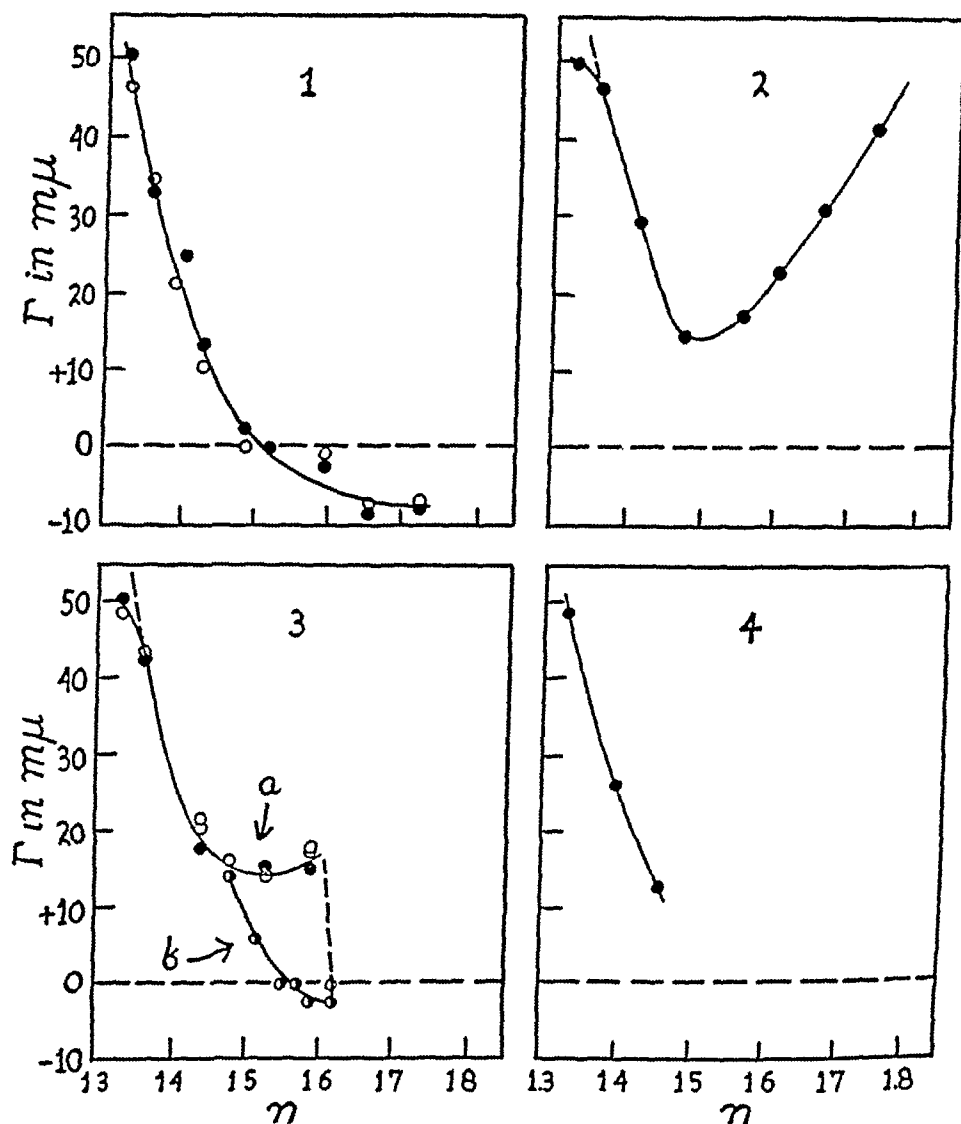
The commonly accepted view of the fine structure of chitin is due to Mohring (1926), who worked with the decalcified inner layer of the lobster carapace Using mixtures of water and mercuric KI, he obtained a U-shaped imbibition curve which twice cut the line of isotropy, passing through a minimum with a residual value of *negative* birefringence when imbibed with fluid of refractive index 1.61 In saturated mercuric KI at $n = 1.73$ birefringence is again positive The interpretation made of this curve is that positive form birefringence is abolished at $n = 1.61$, and the residual negative birefringence is due to the optically negative crystallinity of the chitin micelle Schmidt (1934) has come to similar conclusions from qualitative observations on the treatment of chitinous insect's tendons with mercuric KI solutions X-ray studies (Meyer and Mark, 1930) show that chitin is partially crystalline

Whether or not Mohring's interpretation is correct in theory, its reasonableness depends on the U-shaped character of the imbibition

curve, which twice cuts the line of isotropy. Careful repetition of the mercuric KI imbibition of crayfish chitin yielded a curve (Fig 1) the left hand limb of which resembles Möhring's, but with no trace of a secondary rise at the highest values of n . The reproducibility of the curve is shown by two series of measurements, taken a week apart, plotted in Fig 1. The fact that the curve approaches a limiting value of negative double refraction with the highest concentrations of mercuric KI suggests a progressive, oriented association of ions or molecules of the solution with the chitin micelles, rather than a purely physical effect based on increase of refractive index of the intermicellar fluid.

Imbibition with xylol methylene iodide mixtures gives a curve covering the same wide range, but differing radically from that obtained with mercuric KI solutions. Here the material is passed from water into absolute ethyl alcohol, then into xylol, and then into mixtures of xylol with methylene iodide. The left hand limb of the curve (Fig 2) runs parallel to but somewhat higher than the previous one (Fig 1), and there is evidence that this net increase in double refraction is due to dehydration in the passage from water into alcohol. The remarkable aspect of this curve, however, is that with progressive addition of methylene iodide to xylol it rises sharply to high values of positive double refraction. This U shaped curve, then, could be interpreted to mean that in addition to positive form double refraction chitin possesses a residual positive double refraction of the micelle, and that the crystalline micelle is optically positive.

It is conceivable that the marked difference between the curve for mercuric KI and for xylol methylene iodide mixtures might be due to the fact that in one case the chitin micelle was hydrated, in the other dehydrated. To test this idea, mixtures of another non aqueous liquid, iodobenzene, were made up with (1) absolute ethyl alcohol, and (2) with xylol. The results of imbibing the same piece of chitin with these fluids are shown in Fig 3. The xylol iodobenzene curve continues to fall steeply beyond the point for pure xylol, cuts the line of isotropy at $n = 1.55$ to 1.57 , and gives a very weak negative double refraction in pure iodobenzene ($n = 1.62$). The alcohol iodobenzene curve is anomalous. It reaches a plateau or a slight minimum at $n = 1.53$, and keeps approximately that value in mixtures containing



Imbibition curves of crayfish chitin. Abscissa refractive index of imbibing liquid, ordinate retardation, Γ , in $m\mu$ for blue light. Dotted lines represent isotropy ($\Gamma = 0$).

FIG 1. Mercuric KI imbibition. The top pair of points is for water, all others for water-mercuric KI mixtures. Solid and open circles are separate series.

FIG 2. Points on the left are, reading from the top, for water, ethyl alcohol, alcohol-xytol mixture, xylol. The right-hand limb of the curve is for xylol-methylene iodide mixtures, ending with pure CH_2I_2 ($n = 1.74$).

FIG 3. The top pairs of points are for water, then ethyl alcohol. Curve a, alcohol-iodobenzene mixtures (open and solid circles are separate series), curve b, xylol-iodobenzene mixtures (half-solid circles).

FIG 4. Imbibition with water, water-glycerol mixture, glycerol.

more and more iodobenzene. In pure iodobenzene, however, it falls suddenly to isotropy or slight negative double refraction. This discontinuity may be ascribed to either incomplete penetration or to selective binding of a constituent of the mixture. In any case, the iodobenzene imbibition series show that isotropy and negative double refraction can be obtained with non aqueous fluids as well as with aqueous mercuric KI solutions, so the presence or absence of water cannot be the reason for the different types of curve.

Imbibition with water glycerol mixtures gives the fragmentary curve in Fig 4, which runs approximately parallel to and in between the imbibition curves previously described. Unfortunately the refractive index of pure glycerine is so low, $n = 1.46$, that the course of the curve at the higher, critical values of n cannot be judged.

IV

Which set of data are we to believe represents a normal imbibition curve of the Ambronn type? According to the simplest form of the theory, double refraction due to the parallel arrangement of rod like submicroscopic elements imbedded in a continuous medium of different refractive index is abolished if the continuous phase has the same refractive index as the dispersed phase. The imbibing fluid is assumed to act only by virtue of its refractive index, and must therefore enter into no chemical reactions or oriented adsorptions with the dispersed phase. Relatively inert structures, such as the silicious shells of diatoms or the silicious skeleton of the barley awn (Frey, 1926) exhibit positive form double refraction, and their imbibition curves are of the simple U shaped type demanded by the theory. More complex organized structures, such as the polysaccharides, are well known to enter into a variety of adsorption compounds, and von Ebner (1894) long ago described the association of certain phenols and aromatic aldehydes with collagen, reversing the sign of its double refraction. Chitin also forms such associations, especially with aldehydes. In interpreting the results of imbibition curves of such substances, therefore, great care is necessary, particularly since the choice of fluids having refractive indices above 1.5 is limited to a few.

All of the curves agree in showing a rapid decrease in the double refraction of chitin between $n = 1.33$ and $n = 1.49$. The point for

xylol ($n = 1.49$), a hydrocarbon, seems particularly reliable. There can be little doubt, therefore, but that a very large proportion of the positive birefringence of chitin is due to the form element, vastly more than in the case of cellulose.

The further course of the curve depends wholly on what imbibing fluids are used. The methylene iodide curve (Fig. 2) is pleasing if one hopes to obtain a U-shaped curve, and it may be significant that it can be drawn to inflect near $n = 1.525$ which Becking and Chamberlin (1925) found to be the refractive index of crab and insect chitin. The course of the alcohol-iodobenzene curve (Fig. 3) appears to have a minimum in the same region. Pure iodobenzene, however, produces feeble negative double refraction, and an aqueous mercuric KI mixture of $n = 1.525$ produces isotropy. Since there is no certain way of disentangling the influence of changing refractive index from chemical or adsorption effects, it is impossible at present to favor one of these procedures over another. The complex ions present in the mercuric KI solutions would appear, however, to make use of this reagent particularly unsuitable. The course of the mercuric KI imbibition curve certainly points to an action on chitin beyond that expected merely on the basis of its refractive index. It is unsafe, then, to draw conclusions from such experiments about the crystalline nature of the chitin micelle.

SUMMARY

The double refraction of the chitinous hair of the crayfish is positive with respect to the axis of the hair, and is largely caused by the arrangement of submicroscopic, elongated chitin particles parallel to this axis (*form birefringence*). Using a series of relatively unreactive liquids and fluid mixtures which permeate the chitin framework, the type of curve relating double refraction and refractive index of the imbibed fluid is found to depend greatly on the chemical nature of the fluid. Either a positive or a negative residual birefringence may be found, depending on the choice of imbibing liquid. Separation of form and crystalline elements in double refraction by means of Ambrohn's imbibition technique is therefore unsafe in a system like chitin, where some type of oriented association of the imbibed molecules with the chitin framework is prevalent.

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ON THE INDIVIDUAL CHARACTERISTICS OF ANIMAL AMYLASES IN RELATION TO ENZYME SOURCE

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A great number of methods have been devised for the demonstration of differences in quality of amylolytic agents. A composite difference between enzyme preparations may be demonstrated by an examination of the products of starch digestion. Diffusion, precipitation, and other physicochemical means of separation may furnish similar information. Wijsman¹ in 1889, using a combination of such methods, clearly demonstrated in a series of ingenious experiments that there were two types of amylase in malt extract. These methods were applied and extended by subsequent workers,²⁻⁷ and furnished criteria for classification but no direct evidence of individuality within the classes. Another method to which investigators have had frequent recourse is that of comparing variations in relative activity with change in pH. In order to identify such variations as characteristic of the amylolytic agent from a given source, care must be exercised to avoid uncontrolled variations in other constituents of reaction mixtures which might appreciably affect the relative activity or stability of the enzyme com-

* Davis and Geck Fellow in Surgery. The authors were assisted in the technical work by Mr. Emery F. Barringham, and are indebted to Mr. John F. Iannucci for voluntary aid in an emergency.

¹ Wijsman, H. P., *Rec trav chim Pays bas*, 1890, 9, 1 (also in a dissertation Amsterdam, 1889).

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³ Van Klinkenberg, G. A., *Z physiol Chem*, 1932, 209, 253.

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ponent⁸⁻¹¹ In this way, a striking similarity has been demonstrated¹² between the amylase of human serum and that derived from hog pancreatin (Parke Davis)

It is quite conceivable, however, that in spite of similarity in this respect, a difference between two amylases may be demonstrable in some other way Another basis of comparison is furnished by the course of enzyme inactivation, either spontaneous or induced In certain cases this may be shown to approximate the so called monomolecular type, i e

$$Q = Q_0 e^{-kt},$$

where Q is the enzyme concentration at the time, t , and k is a constant called the *speed coefficient* Otherwise stated, the logarithm of the enzyme concentration is a linear function of time With close approximation, this has been shown to hold for inactivation of hog pancreatin by ultraviolet radiation,¹³ and Giri,¹⁴ using a commercial pancreatin preparation, indicated the same course for the early stages of spontaneous inactivation When the course of inactivation is monomolecular in type, the speed coefficients may be used as a basis of comparison of two enzyme preparations from different sources, provided that all other conditions which influence stability are maintained the same One of the best methods of insuring such control, particularly when some of the factors are unknown, is to have the enzyme component from each source present in the same solution If the enzyme supplied from both sources is the same individual, then the course of the combined inactivation will still be monomolecular in form This might also obtain with a mixture of enzymes which happen to have the same stability under the experimental conditions, but otherwise, as has been found¹⁵ in irradiation of mixtures of yeast invertase and malt amylase, the logarithm of the combined enzyme

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¹¹ Ambard, L, and Trantmann, S, *Compt rend Soc biol*, 1933, 112, 1532

¹² Thompson, W R, Tennant, R, and Wies, C H, *J Biol Chem*, 1935, 108, 85

¹³ Thompson, W R, and Hussey, R, *J Gen Physiol*, 1931-32, 15, 9

¹⁴ Giri, K V, *J Indian Inst Sc*, 1932, 15A, 117

¹⁵ Chauchard, A, and Mazoué, B, *Compt rend Acad* 1911, 152, 1709

concentration¹⁶ will not be a linear function of time, in which case differences from linearity should be most readily detected if the initial enzyme contributions from the two sources are equally potent. Thus a monomolecular inactivation course furnishes strong indication that a single *chemical individual* is responsible for the enzymic activity.

The purpose of the present communication is to present the results of experiments in which amylases from various animal sources were investigated with the primary object of obtaining evidence as to whether the amylolytic activity observed under our experimental conditions could be attributed in a given instance to a single *chemical individual*, and whether we could recognize the same individual in preparations from other sources.

Technique

Amylolytic activity was estimated by a viscosimetric method of high precision which has been described elsewhere,¹² except that a convenient modification of substrate preparation, subsequently reported,¹⁷ was used unless otherwise stated. The activity indicated, sometimes called the *starch liquefying power*, reflects chiefly the rate of breakdown of the larger molecules in the substrate, principally α amylose, since further reactions in the cascade ending in sugar formation involve relatively little viscosity change. Accordingly, conditions which effect uncontrolled variations in the rates of these subsequent reactions introduce relatively little error in the estimation of amylolytic activity. Obviously, amylases which act only or principally on these later degradation products¹⁸ are outside the province of the present report.

In studies of spontaneous inactivation (sometimes called heat inactivation) the enzyme solutions were made to contain 5 gm of CaCl_2 per 100 ml in addition to the small and variable contribution from the enzyme source. This was done because Ca ion in low concentration is known to influence the rate of spontaneous inactivation,⁸ and it was hoped that introduction of a large excess would diminish the influence

¹⁶ On the basis of measurements of activity under given conditions, enzyme concentrations may be expressed in terms of equivalent units and thus combined concentrations may be represented by Q , as in the case of a single enzyme.

¹⁷ Thompson, W. R., *J. Biol. Chem.* 1935, 109, 201.

¹⁸ Such character has been attributed to the so called β amylases.

of the small variable fraction. As these inactivation rates varied greatly with the pH, an acetate buffer system was used, adjusted to a pH such that the course of inactivation could be followed conveniently. The temperature used throughout these studies was approximately 37.5°C with an extreme variation of about 0.1° during the course of any given experiment.

In the preparation of inactivation mixtures, the enzyme components were maintained until warmed to the required temperature at a pH such that they were relatively stable. Dilution to the required volume and a shift of pH to the acid side was then accomplished without danger of momentarily overshooting the mark by rapid addition with vigorous stirring of a quantity of separately warmed solution containing buffers and other salts as required for the final solution (*E*) whose inactivation course was to be followed. The time of the beginning of inactivation was taken as that when half of this last component had been admixed.

For the estimation of *Q* at different times during the course of the reaction, the conditions in the ordinary use of the substrate, *S*₂₂, were approximated.¹² Under these conditions, the remaining enzyme was stabilized. The procedure involved transfer of samples of *E* to portions of specially prepared substrate, and the concentration of amylase so estimated was taken to apply to *E* at the time when half the sample had been added.

In the ordinary use of *S*₂₂, *x* parts of enzyme solution are added to (4-*x*) parts of saline (0.85 per cent NaCl) plus 20 parts of *S*₂₂. In experiments of the type mentioned above, *x* parts of the enzyme solution (*E*) were added to (4-*x*) parts of *D* plus 20 parts of *S'*, where *D* was a solution containing the same amount of CaCl₂, acetates, and NaCl as added per unit of final volume in preparation of *E*. *S'* was the same as *S*₂₂ except that the concentrations of CaCl₂, acetic acid, and sodium acetate were diminished by one-fifth those of *D*. The technique of preparation of *E* was varied to meet peculiar requirements of individual experiments, e.g., when serum was included it was convenient to compensate approximately its tendency to induce pH shift by inclusion of an amount of HCl determined by preliminary titration of a portion of serum as in previous experiments.¹² In any case, the activity was estimated under comparable conditions throughout a given experiment, and expressed in terms of *Q*, the concentration of hog pancreatic amylase of equal potency under the same circumstances. This was done for convenience in expression of results of the present experiments.

EXPERIMENTAL

The first experiment dealt with the spontaneous inactivation of a solution of hog pancreatin (Park Davis) and one of a mixture of this with human saliva, which were prepared as follows

A suspension of 0.1 gm. of the pancreatin in 100 ml. of 0.85 per cent NaCl was allowed to stand at room temperature for a half hour, filtered, and stored in a refrigerator. 4 ml. of this were transferred to a 100 ml. volumetric flask, and to this were added 50 ml. of a solution, *B*, containing 50 gm. of CaCl_2 , 0.18 moles of sodium acetate, and 0.02 moles of acetic acid per liter. This diluted to volume with distilled water¹⁹ formed a preliminary solution, E'_1 . Another solution, E_2 , was made similar to E'_1 with the exception that 2 parts per 1000 of human saliva (W. R. T.) were included instead of the pancreatin extract. By trial under the standard digestion conditions, it was found that E_1 had approximately 44 per cent of the amylolytic power of E_2 . Accordingly, E_3 was made by dilution of 44 parts by volume of E_2 to 100 parts with a solution, $B/2$, made by dilution of a portion of Solution *B* to double volume with water. Thus, E_3 and E'_1 were respectively solutions of human saliva and of hog pancreatin of conventionally equal potency. A solution, *D*, was prepared also, consisting of 25 parts of Solution *B* plus 8 parts of molar acetic acid, all diluted with water to a total volume of 100 parts. The inactivation mixture E_1 was then made by addition with vigorous stirring of a portion of *D* to an equal portion of E_1 , both solutions being at 37.5°C. A solution, E_2 , was made similarly with a mixture of equal parts of E'_1 and E_3 being used instead of E_1 alone.

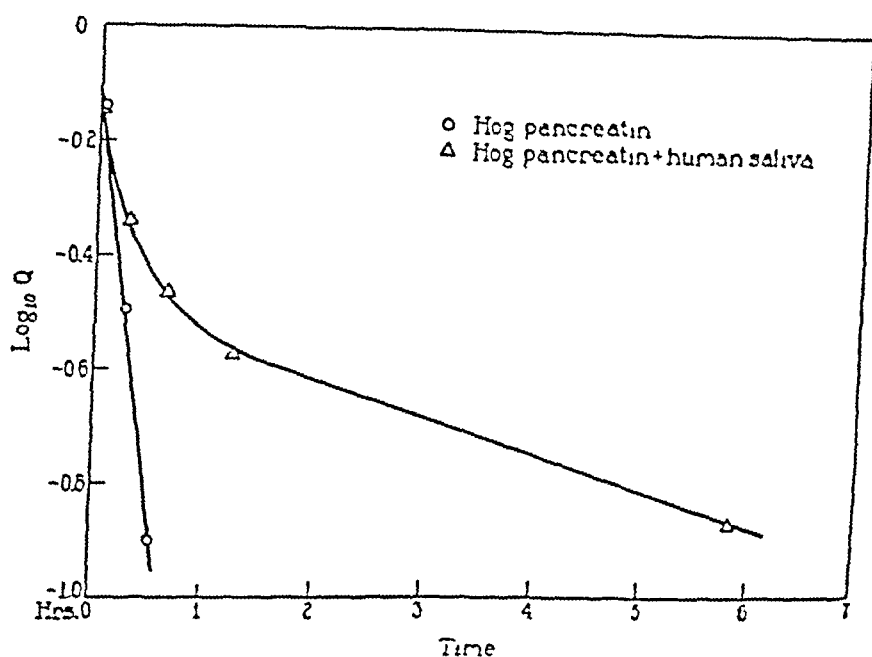
The spontaneous inactivation course for the hog pancreatin solution E_1 , was of the characteristic monomolecular type, as may be seen in Text fig. 1 from the linear relationship between the logarithm of *Q* and time. The course for the hog pancreatin human saliva mixture, E_3 , however, is not of this type, indicating the presence of more than one amylolytic substance.

Similar experiments were performed with the serum of a depancreatized dog, alone and in mixtures with human saliva and with hog pancreatin. The concentrations of CaCl_2 and acetates *as added* were the same as in E_1 above, and in each case the same concentration of the same serum was used, so that the three experiments would be comparable with regard to any shift in pH induced by the serum component. The results are presented in Text fig. 2, where it may be seen that while dog serum alone followed the course of monomolecular

¹⁹ Water redistilled in glass was used in all reagents

inactivation, neither of the mixtures did. The amylolytic agent of the serum of a depancreatized dog appears thus to act as a single substance as does that of hog pancreatin, but the two are strikingly different from each other and from the amylolytic component of human saliva.

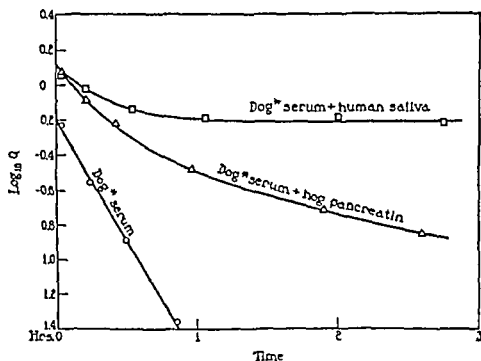
Of the enzymes investigated in the preceding experiments, the amylase of hog pancreatin and that of the serum of a depancreatized dog individually pursued a monomolecular inactivation course. When



TEXT-FIG. 1 Spontaneous loss of amylolytic potency of hog pancreatin and of a mixture of hog pancreatin and human saliva

human saliva was the sole source of amylase, the course of inactivation resembled the monomolecular type except for the early portion, which exhibited a characteristic discrepancy, apparently greater the more dilute the solution of saliva. This and certain other peculiarities were brought out in experiments in which two or more solutions, differing only in initial concentration of saliva from the same specimen, were followed simultaneously. With tentative exclusion of the first observation as a prescribed condition, a straight line was fitted in each case to the observed coordinate points ($t, \log Q$) so as to minimize the sum

of squares of deviations of $\log Q$ from the fitted line. For convenience in graphic presentation, Q_0 was taken as the antilog of the initial ordinate of this line (the calculated Q for $t = 0$), and the results were replotted with $\log \frac{Q}{Q_0}$ as ordinate instead of $\log Q$. This is equivalent to lowering all points and the fitted line for a given inactivation experiment by the constant, $\log Q_0$, thus making the fitted lines for the different experiments all initiate from the same point on the graph. Results are given in Text-fig. 3 of two sets of experiments with speci-

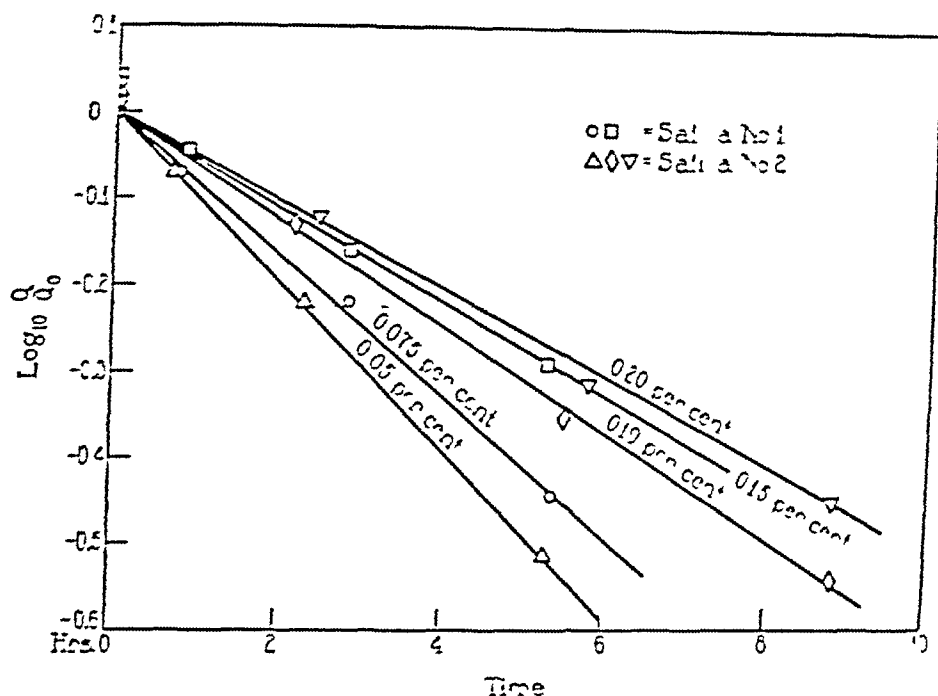


TEXT FIG. 2 Spontaneous loss of amylolytic potency of the serum of a depancreatized dog* and of mixtures of this with human saliva and with hog pancreatin

mens of saliva from the same individual, where two solutions of specimen 1 were compared and three solutions of specimen 2, having initial saliva concentrations (C) successively halved. A progressive diminution of relative inactivation rates with increase in C is evident in the figure, and also in Table I, where the speed coefficient (k_0) is given in each case for the fitted line, $\log \frac{Q}{Q_0} = -k_0 t$. It is interesting to note that $k_0 \sqrt{C}$ is about the same throughout. An indication of higher initial inactivation rates than would be found in reactions of the

*Depancreatized 10 days previously

monomolecular type with the same later course is given by the fact that the first observed point in each case is above the straight line fitted



TEXT-FIG 3 Spontaneous loss of amylolytic potency of solutions of human saliva

TABLE I

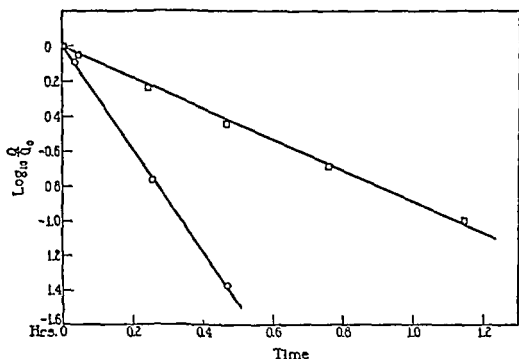
Relation of Initial Concentration (C) of Human Saliva to the Fitted Speed Coefficient (k) for Spontaneous Loss of Amylolytic Potency, and to the Extrapolated Q_0

Specimen No	Saliva concentration $C = C(\%)$	k	$k \sqrt{C}$	Extrapolated Q_0	Q_0/C
1	15.0	0.1258	0.497	0.8926	0.595
1	7.5	0.1874	0.513	0.3996	0.533
2	20.0	0.1185	0.530	2.062	1.031
2	10.0	0.1423	0.450	0.6670	0.667
2	5.0	0.2236	0.500	0.4397	0.879

to the remaining points, as illustrated in Text-fig 3. Moreover the extrapolated values of Q_0 give ratios, Q_0/C , which may be seen in Table I to decrease successively with C , the initial saliva concentra-

tion²⁰ This points to a rapid destruction of a small portion of the enzyme initially present, relatively more marked the greater the dilution

The possibility of distinguishing animal amylases from different sources by studying the course of inactivation of enzyme mixtures having been established, we thought to apply this technique to the elucidation of a problem with which we were concerned In a series of experiments to be presented elsewhere,²¹ we found that, in dogs sub



TEXT FIG 4 Spontaneous loss of amylolytic potency of two mixtures of serum of a depancreatized dog and dog pancreatic extract The first of these experiments described in the text is represented by the lower line

jected to pancreatectomy and maintained with insulin, the amylolytic power of the serum fell to about half the preoperative level and seemed

²⁰ In the experiments of Text fig 3 the initial Q was not determined experimentally, but an observation was made in each case as soon as practicable after inactivation was initiated Had the peculiar results been anticipated we would have had recourse to a device employed in subsequent experiments In these (Text figs 4 and 5) Q_0 is an observed value determined by addition to a digestion mixture of the same components initially introduced into the inactivation mixture, but in a different order (enzyme last) so as to avoid spontaneous inactivation

²¹ Friedman, I, and Thompson, W R., *Ann Surg*, in press

fairly well stabilized at that point for long periods of time (4 months). Apparently the pancreas is not the sole source of serum amylase, and we were eager to determine whether or not the enzyme supplied from other sources could be distinguished from that derived from the pancreas. Accordingly, an enzyme mixture was prepared containing equipotent contributions from the serum of a depancreatized dog and a saline extract of fresh dog pancreas. The inactivation course was followed as in the previous work, but due to an error in preparation the concentrations of acetates and of CaCl_2 in the inactivation mixture were all 1.087 times the usual values. This did not invalidate the experiment, but it was nevertheless repeated under the standard conditions. In each case the inactivation course is that of a monomolecular reaction, as may be seen in Text-fig. 4. The sera used in these experiments were obtained from two different dogs on the 16th and 133rd postoperative days, respectively. The possibility that some pancreatic tissue had been left inadvertently at operation was investigated postmortem, but careful examination failed to reveal any remnant.

We turned next to an investigation of amylases in man, where a third convenient source of enzyme, the saliva,²² is available for study. Elman and others²³⁻²⁵ have indicated the value of serum amylase determination in the diagnosis of certain disorders of the pancreas. The possibility of an extrapancreatic supply of amylase to the blood stream is therefore of considerable clinical importance. The experiments which follow were designed to determine whether or not the kinds of amylase found in human saliva and human pancreas are distinguishable, and what resemblance that of serum might bear to each. Accordingly, an equipotent mixture of human saliva and saline extract of fresh human pancreas was prepared, and inactivation of the enzyme was followed in a solution containing 5 gm. of CaCl_2 per 100 ml.

²² In the case of the dog, saliva and saline extract of fresh extranguinated salivary gland were found to have no appreciable amylolytic power under our experimental conditions.

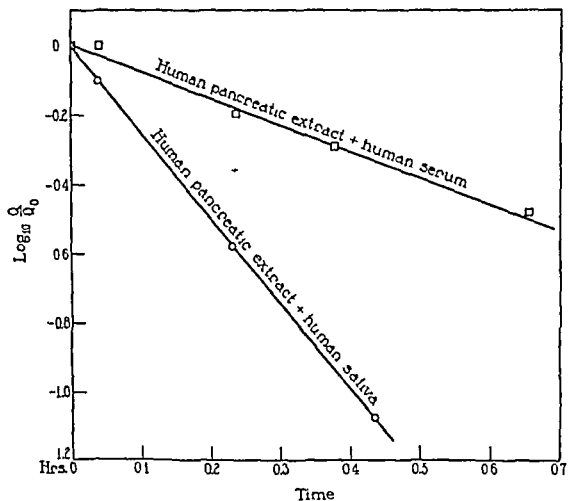
²³ Elman, R., *Proc. Soc. Exp. Biol. and Med.*, 1927-28, 25, 173.

²⁴ Elman, R., Arneson, N., and Graham, F. A., *Arch. Surg.*, 1929, 19, 943.

²⁵ Wakefield, E. G., McCaughan, J. M., and McVicar, C. S., *Arch. Int. Med.*, 1930, 45, 473.

²⁶ Elman, R., *Arch. Int. Med.*, 1931, 48, 828.

as before, but with a total acetate concentration of 0.15 moles per liter, half of which was supplied as acid and half as sodium salt. A similar mixture was made with human serum instead of saliva and with an amount of HCl included, which was estimated roughly to compensate the tendency of serum to shift the pH toward the alkaline side. In each case a monomolecular type of inactivation occurred. The results are given in Text fig. 5.



TEXT FIG. 5 Spontaneous loss of amylolytic potency of mixtures of human pancreatic extract with human serum and with human saliva.

In previous work,¹² to which reference has been made above, human serum amylase was found to bear a striking resemblance to that of hog pancreatin with regard to variation in activity²⁷ with change in pH.

²⁷ The present methods¹²⁻¹⁷ of estimation of amylolytic activity are based on determination of the time ω , since the beginning of digestion, such that a 7.5

in the range (5.1, 5.6). A study similar to these was made with human saliva, with the result given in Table II, where R is the ratio of activity with the substrate, S_{55} , to that with $S_{19.8}$, freshly autoclaved preparations being used as in the earlier work. Six experiments with saliva from five different individuals gave a mean $R = 0.613$ (with an

TABLE II

Amylolytic Activity of Human Saliva with the Substrate, S_{55} , Relative to That with S_{22}

Subject	R
W R T	0.633
W R T	0.633
S M I	0.583
J F I	0.639
J R	0.596
E L I	0.595
Mean = 0.613	
a d = 0.022 = 3.6 per cent of mean	
σ = 0.024	
σ_m = 0.010	

TABLE III

Variation in Activity of Hog Pancreatic Amylase with Change in pH, As Indicated by the Values of ω Obtained with Different Substrate Systems

Experiment	S_{11}	$S_{11.4}$	$S_{11.8}$
	ω	ω	ω
1	0.721	0.587	0.412
2	0.714	0.584	0.430
3	0.728	0.582	0.410
Mean	0.721	0.584	0.424
a d	0.005	0.002	0.003
Relative values	1.000	0.810	0.563

which is sensibly the same as the
of about 1.5 per

cent in each case) previously obtained with human serum and hog pancreatin, respectively

In further studies of this nature, the modified substrates¹⁷ were used because of their greater convenience. In order to see if this introduced a considerable difference, the hog pancreatin experiment was repeated with the modified substrates, with the results given in Table III. The same solution of hog pancreatin was used in three experiments, and the close agreement between values in any given column indicates that the preparation was fairly stable. The relative values of the mean ω , calculated in the usual way,²⁷ for the three substrates were 1.000, 0.810, and 0.588, whereas those obtained with

TABLE IV

Variation in Activity of Dog Pancreatic Amylase with Change in pH, As Indicated by the Values of ω Obtained with Different Substrate Systems

Experiment	S ₁₀	S _{7.4}	S _{6.8}
	ω	ω	ω
1	0.854	0.663	0.446
2	0.896	0.673	0.444
3	0.877	0.681	0.452
4	0.854	0.663	0.438
Mean	0.870	0.670	0.445
s.d.	0.011	0.007	0.004
Relative values	1.000	0.770	0.511

the freshly autoclaved substrates¹² were 1.000, 0.824, and 0.621. Studies were made of the variation of relative activity of dog amylases with change in pH, in which the modified substrates were used under the conditions of the hog pancreatin experiment above, in order to see if similar ratios would be found. The results obtained with a saline extract of fresh dog pancreas, serum of a depancreatized dog, and sera of three normal dogs, are given, respectively, in Tables IV, V, and VI. Relative values are given in the last table in order to facilitate comparison of the experiments, since the three sera were not of equal potency. The average relative values given in the last three tables are in close agreement with each other, but differ from those obtained with hog pancreatin.

Thus the amylase preparations, indistinguishable on the basis of spontaneous inactivation of mixtures, exhibited sensibly the same activity-pH shift relations, in so far as these were studied. The agreement in this respect between the hog and human amylases is curious.

TABLE V

Variation in Activity with Change in pH of the Serum Amylase of a Depancreatized Dog, as Indicated by the Values of $a\omega^$ Obtained with Different Substrate Systems*

Experiment	S ₅₅	S _{77.4}	S _{79.2}
	$a\omega^*$	$a\omega^*$	$a\omega^*$
1	1 276	0 966	0 634
2	1 269	1 001	0 624
3	1 214	0 957	0 638
Mean	1 253	0 975	0 632
a d	0 019	0 018	0 005
Relative values	1 000	0 777	0 504

* The same serum was used in the three experiments, but the concentration in the first was twice that of the others. Accordingly, $a = 1$ or 2 , proportional to the serum concentration, is introduced to facilitate comparison.

TABLE VI

Variations in Activity with Change in pH of the Serum Amylase of Normal Dogs, As Indicated by the Relative Values of ω Obtained with Different Substrate Systems

Dog	S ₅₅	S _{77.4}	S _{79.2}
	ω/ω'^*	ω/ω'^*	ω/ω'^*
1	1 000	0 781	0 523
2	1 000	0 782	0 519
3	1 000	0 788	0 506
Mean relative values	1 000	0 784	0 516

* In each case, ω' is the value of ω obtained with S₅₅ and the same serum.

in view of the difference demonstrated by spontaneous inactivation studies. Thus, while the enzymes are almost certainly not identical, this striking similarity suggested the possibility that a common amylolytic radical might be involved. It appeared as a possibility that in

different substances this radical might be equally sensitive to ultra violet light, which is known to induce a monomolecular type of inactivation of hog pancreatic amylase. This stimulated us to study such inactivation of hog and human amylases. Mixtures of these amylases were used since in no other way could we control conveniently the effects of protective action^{28, 29} in addition to other conditions affecting stability as in the spontaneous inactivation studies. Obviously, these conditions should be such as to make the enzymes fairly stable except for the irradiation effects.

An equipotent mixture of human salivary and hog pancreatic amylases was used in solution with 5 gm. of CaCl_2 per 100 ml. and a total acetate concentration of 0.1 m p l., one tenth of which was introduced as acid and the rest as sodium salt. The conditions of irradiation were essentially the same as described in previous communications^{13, 28}. Briefly summarized, these were as follows:

The radiation source was a mercury arc in quartz operated at approximately 75 volts and 4.0 amperes. Above this was a distilled water bath at $10.0^\circ \pm 0.1^\circ\text{C}$, provided with a quartz window in the bottom through which a beam of radiation passed, and was filtered through 5 mm. of the bath water before reaching the flat bottom of a quartz tube, containing 5 ml. of the solution to be irradiated. This solution and the bath water were maintained independently in a state of vigorous agitation during the period of irradiation.

Five experiments were performed in each of which the calculated speed coefficient, k_1 , for a 7 minute irradiation was compared with that for a 14 minute irradiation, k_2 . These periods were chosen on the basis of preliminary trial with the object of inactivating approximately one half and three quarters, respectively, of the enzyme present. The pairs of irradiations in each experiment were done in rapid succession in order to avoid gross errors which might be introduced by gradual changes in protective properties of the solution or in the mean intensity of effective radiation such as have been discussed in previous reports^{13, 28, 30}. The results are summarized in Table VII where the existence of some such

²⁸ Thompson, W. R., and Tennant, R., *J. Gen. Physiol.* 1934-35 18, 675

²⁹ Thompson, W. R., and Tennant, R., *Proc. Soc. Exp. Biol. and Med.* 1932, 29, 510

³⁰ Hussey, R., and Thompson, W. R., *J. Gen. Physiol.*, 1925-26 9, 217

phenomenon is suggested by the general drift toward lower values of k . However, the approximation to unity of the ratios, $\frac{k_1}{k_2}$, indicates that the combined inactivation of the two amylases follows the course of a monomolecular radiochemical reaction

TABLE VII

The Course of Inactivation Induced by Ultraviolet Irradiation of a Mixture of Human Salivary and Hog Pancreatic Amylases

Experiment	Q_0	$\frac{Q_1}{Q_0}$	$\frac{Q_2}{Q_0}$	k_1	k_2	$\frac{k_1}{k_2}$
				(min) ⁻¹	(min) ⁻¹	
1	0 851	0 548	0 291	0 0862	0 0882	0 977
2	0 870	0 558	0 294	0 0836	0 0876	0 955
3	0 833	0 621	0 357	0 0683	0 0738	0 925
4	0 797	0 589	0 364	0 0757	0 0723	1 047
5	0 826	0 616	0 428	0 0693	0 0606	1 143

Mean = 1 009

σ = 0 087

Note Q_1 and Q_2 are the values of Q after 7 and 14 minute irradiations, respectively, and $k = \frac{1}{t} \log_e \frac{Q_0}{Q}$

DISCUSSION

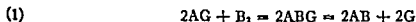
The usual classification of α and β -amylases has been based, unfortunately for our purposes, on the products of starch digestion² rather than on the amylose substrates upon which the enzymes act. From studies of certain enzymes of these two types the suggestion has been advanced that α -amylases are able to act upon α -amylose, whereas β -amylases are not. Such statements must remain unverified, however, as it is not known how many α - or β -amylases may exist, nor that the classes are mutually exclusive. As noted in the introduction, the amylolytic action measured in the present work may be considered to reflect essentially the breakdown of α -amylose, and it should be clearly understood that the present discourse is concerned only with enzymes having such potency. This action is usually ascribed to α -amylases, but, for the reasons given, we are reluctant to use this nomenclature. Obviously, the controversy between Giesberger⁵ and Van Klinkenberg⁴

as to presence of β amylase in the animal body is beyond the scope of the present investigation

In the case of hog pancreatin, a strong indication that the amylolytic action is due to a single chemical individual is given by the monomolecular character of the spontaneous inactivation course. The same is true of serum of a depancreatized dog. As has been pointed out above, however, the spontaneous inactivation course with human saliva presented certain discrepancies from the monomolecular type, relatively less marked the greater the initial saliva concentration (C), and imperceptible in mixture with human pancreatic extract. This behavior is more easily reconciled with the postulation of a single amylase subject to reversible dissociation, than of a mixture of independent amylases of different stability. A reversible dissociation may account likewise for the observed approximate inverse proportionality between \sqrt{C} and the fitted speed coefficient (k_0). The postulation of side reactions should not appear strange, since, in the case of spontaneous inactivation of trypsin, such a reaction has been shown by Northrop²¹ to account for discrepancies from the monomolecular course.

A possible explanation of the observed phenomena may be based on the following assumptions

A, B, and G are radicals, and ABG is an amylolytic substance involved in reversible reactions of the type,



such that, in accord with the law of mass action, approximately,

$$(2) \quad \frac{C_{AG}^2 C_B}{C_{ABG}^2} = K \text{ a constant}$$

ABG is otherwise stable, and only a *small* portion of the total A exists at any time in the forms, AB and AG, which rapidly undergo irreversible destruction with loss of A at rates proportional to the amount present. The concentration of B_2 is great enough at all times to be relatively little influenced by the reaction (1), and is proportional to C , the initial saliva concentration. The concentration of G is initially *small*, and is relatively greatly influenced by the reaction on the right

²¹ Northrop J. H. *J. Gen. Physiol.* 1921-22 4, 261

of (1), so that AB soon becomes negligible as a means of destruction of A

It follows from these assumptions that approximately

$$(3) \quad C_{AG} = \frac{K'}{\sqrt{C}} C_{ABG},$$

where K' is a constant After loss of A through AB has become negligible, then

$$(4) \quad C_A = C_{AG} + C_{ABG}, \text{ approximately,}$$

where C_A is the *total A concentration* Substituting (3) in (4) and differentiating with respect to t , then

$$(5) \quad \frac{dC_A}{dt} = \left(1 + \frac{K'}{\sqrt{C}}\right) \frac{dC_{ABG}}{dt}$$

But, under these conditions,

$$(6) \quad \frac{dC_A}{dt} = K'' C_{AG},$$

where K'' is a constant, whence (3) and (5) give

$$(7) \quad \frac{dC_{ABG}}{dt} = \frac{K' K''}{\left(1 + \frac{K'}{\sqrt{C}}\right) \sqrt{C}} C_{ABG} = \frac{K' K''}{K' + \sqrt{C}} C_{ABG}$$

This describes a monomolecular reaction course, whose speed coefficient (k_0) is the coefficient of C_{ABG} on the right of (7) It is obvious that when K' is *small* relative to \sqrt{C} , as follows from (3) and the fundamental assumptions, then k_0 is approximately inversely proportional to \sqrt{C}

However, under conditions where K' is not small in comparison with \sqrt{C} , the model demands that with given changes in C relative variations in k_0 be in the same direction as before, although not as great It is obvious also from (7) that under these conditions the speed coefficients would be greater The counterpart of this hypothetical situation in regard to relative variation of k_0 with respect to \sqrt{C} appeared in fragmentary experiments similar to those of Table I but at a lower pH where corresponding speed coefficients were greater

The reaction follows the monomolecular course described by (7) only after loss of A through destruction of AB has become negligible, as the result of the increased concentration of G. Before this condition is attained, there is a discrepancy from the monomolecular course, characterized by excessive early inactivation rates, relatively more marked the lower the initial saliva concentration, C .

Thus, the model system exhibits the peculiarities observed in our experiments with saliva. The absence of an initial discrepancy with the mixture of human saliva and human pancreatic extract is readily explained in terms of this model by the assumption that the pancreatic extract contains an excess of G. It would be difficult to account for this monomolecular character were the presence of *independent* amylases responsible for the discrepancies observed when saliva was the sole enzyme source. The assumption, however, of the presence of *dependent* amylases could readily be made without greatly altering the model, by assigning amylolytic potency to AB or AG or both. In any case, it appears that the observed amylolytic action of human saliva is principally, if not entirely, attributable to a chemical individual, and that the same individual is responsible for the amylolytic action of human pancreatic extract.

In the studies of spontaneous inactivation of mixtures of serum of a depancreatized dog and dog pancreatic extract, represented in Text-fig 4, the course of reaction was monomolecular in two experiments performed under slightly different conditions, but the speed coefficients differed. Alone, each of these experiments indicates that only one amylase is involved, or several of equal stability under the given conditions. The second alternative is rendered less attractive in view of the combined evidence of the two experiments, since it would be necessary to hypothecate not only equal stability under one set of conditions, but the same change in stability of each of these enzymes with a change in conditions. The experiments demonstrate also the profound influence upon speed coefficients of relatively slight changes in reaction mixtures, and indicate that precise control would be required were enzymes from different sources compared on the basis of speed coefficients separately determined. This is borne out by the experiments of Text fig 5, in which amylase derived from human pancreas was compared successively with enzyme indistinguishable

from it although derived from two other sources. The inactivation mixtures differed in that one contained serum and the other saliva, and, although an attempt was made for the sake of convenience to approximate the same conditions with regard to stability of the enzyme, a marked difference in speed coefficients resulted. However, this in no way mars the experiments individually, and two enzymes, thus indistinguishable from a third, are presumably indistinguishable from each other.

The activity-pH shift data were analyzed statistically, with $x = \log R$ used as variate, where R is the activity with S_{56} divided by that with S_{19} in digestion mixtures made with the same proportion of the same enzyme solution. An analysis of variance was made according to the methods described by R. A. Fisher³². In this way, it was indicated that the mean x for the human saliva experiments of Table II did not differ significantly from the mean derived for hog pancreatin and human sera in the experiments previously reported¹². The variance was significantly greater, being beyond the 1 per cent level of significance in the usual sense. These previously reported experiments with hog pancreatin and human serum utilized a freshly autoclaved substrate¹². The mean x under these circumstances was found to differ slightly but significantly from that obtained with hog pancreatin and the modified substrates in Table III. In this case Fisher's t -test gave $P \cong 0.0002$. The same test applied to results of experiments in Tables IV, V, and VI with dog pancreatic extract, serum of a depancreatized dog, and sera of three normal dogs, where the modified substrates were used, revealed no significant difference between corresponding mean values, but their pooled mean differed significantly from the mean obtained with hog pancreatin under the same conditions. In this case the t -test gave $P < 0.0001$.

The agreement in character between human serum and salivary amylases supports the evidence of the inactivation experiments represented in Text-fig. 5. On the other hand, the agreement with hog pancreatin is remarkable in view of the apparent non-identity of the enzymes, and is fortunate from the point of view of evaluation of relative amylase concentration in that, if a unit is established for one of these enzymes, the concentration of the others may be expressed in

³² Fisher, R. A., Statistical methods for research workers, London, Oliver and Boyd, 5th edition, 1934.

terms of equivalence independent of the pH (within the range studied) at which a comparison is made. It is interesting to note in this regard fragmentary evidence of similarity in relative activity of hog and human pancreatic extracts at pH 5.1 and at pH 6.7 with a different substrate system. The mean relative potency for two experiments as estimated at pH 5.1 differed by 2.4 per cent from a similar estimate at pH 6.7.

The apparently equal sensitivity of hog and human amylases to ultraviolet radiation has been supposed the result of possession of a common radical which is responsible for their amylolytic action. It is consistent with our knowledge of photochemical reactions to suppose that the principal destructive action on the enzymes considered is induced by means of a resonance effect on a common radiosensitive radical, exerted suddenly in the case of a given molecule although only at a random coincidence of fortuitous circumstances, the likelihood of a similar result being nearly the same for another molecule having the same radiosensitive radical but certain minor or possibly major differences in the rest of its structure. However, under conditions where *heat* inactivation is studied, the molecule may be subject to a more general attack, and vulnerability may be greatly influenced by even minor differences in molecular structure.

There has been a widespread tendency evident in the literature in the use of expressions such as *pancreatic amylase* and *serum amylase* to emphasize *organ* rather than *species* source. This would seem to imply that the character of amylase is determined by the organ from which it is derived regardless of species. On the contrary, in the domain covered by the experiments of the present report, specification of species source was necessary and sufficient.

SUMMARY

Enzymes from various animal sources responsible for amylolytic activity as measured by a precise viscosimetric method have been investigated with regard to individual characteristics in relation to enzyme source. The principal criteria have been the course of spontaneous inactivation of preparations from different sources alone and in mixtures, and comparison of variations of relative activity with change in pH.

It is strongly indicated that the observed amylolytic activity of hog

pancreatin, and that of the serum of a depancreatized dog are attributable to single chemical individuals, while that of human saliva is caused principally if not entirely by a single individual, which appears to be subject to reversible dissociation

These three individuals are clearly distinguishable from each other

The amylolytic activity of dog pancreatic extract is due to the same individual found in the serum of a depancreatized dog, while that of human pancreatic extract and of human serum are due to the same chemical individual found in human saliva. Thus it may be said of the amylases studied, that specificity depended upon species source rather than organ source

Evidence of similar variations in activity with change in pH and equal sensitivity to ultraviolet light furnish strong indication that hog and human amylases have a common amylolytic radical

THE FORMATION OF MILK SUGAR

THE IN VITRO SYNTHESIS OF LACTOSE BY ACTIVE MAMMARY GLAND PREPARATIONS*

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INTRODUCTION

The question of the origin of milk sugar is undoubtedly one of the most interesting problems of carbohydrate metabolism. As lactose is found only in milk, it is of interest to determine the mechanism of lactose synthesis in the body.

There are three problems which must be considered. First, the place of synthesis, second, the precursors from which lactose is formed, and third, the agency of synthesis.

Bert (1) in 1884 appears to have been one of the earliest physiologists to study the place of synthesis of lactose. He advanced the following hypotheses: (1) The lactose was formed from constituents stored in the mammary gland similar to the storage of glycogen in the liver, or, (2) the lactose was formed by the blood and secreted in the mammary gland. To test out the latter hypothesis Bert conceived the idea of removing the mammary glands before pregnancy and then examining the urine for sugar during pregnancy and after parturition. This experiment was performed on two goats. Tests of the urine during pregnancy showed no trace of sugar, whereas following parturition considerable amounts of glucose were detected during a period of 3 or 4 days. Bert concluded that the milk sugar is formed in the mammary gland from sugar of the blood liberated in excess—probably from the liver—after parturition.

Other experiments of this same type on guinea pigs by Bert gave

* Presented in part before the American Physiological Society Forty-Sixth Annual Meeting, New York, 1934 (*Am J Physiol*, 1934, 109, 108).

negative results Evidence of a negative character was also obtained by Marshall and Kirkness (2) and Moore and Parker (3)

Foa (4) removed the mammary glands of goats and found a hyperglycemia The operations lasted 7 hours However, as operative procedures under anesthesia cause an increase in the blood sugar content associated with a reduction or impairment of renal function, Foa's experiment cannot be considered critical

In a series of papers Porcher (5) reports further study concerned with the origin of lactose He repeated the experiment of Bert but tested the urine excreted by the experimental goats for the presence of both glucose and lactose He confirmed the results of Bert that following parturition there is a glycosuria but the sugar content was observed to decline very rapidly The sugar present in the urine was found to be glucose However, on the 2nd and 3rd day a small amount of lactose was found and upon observation of the animals it was found that some gland tissue was present in spite of the precautions at the time of operation Porcher concluded that the active mammary tissue changes the glucose which is brought to it by the blood into lactose

Gowen and Tobey (6) have shown that the dry udder of the dairy cow is entirely free of reducing sugar

The normal sugar found in the blood is glucose While lactose may be found in the blood it is quickly eliminated by the kidney as a foreign sugar The recent work of Hayden (7) indicates that a considerable amount of lactose is present in the blood of lactating dairy cattle following the inflation of the udder with air in the course of treatment for milk fever

The milk sugar, lactose, is a combination of glucose and galactose The glucose is present in the blood and unquestionably is the precursor of the glucose of lactose The source of the galactose requires further consideration An examination of the foods of herbivorous animals by Muntz (8) indicated that these animals consumed enough galactose-containing compounds to account for the galactose portion of the lactose which they formed However, women and carnivorous animals consume insufficient galactose-yielding substances to account for the lactose which they produce

A further objection to the theory that galactose is brought pre-

formed to the mammary gland is the observation of Porcher (5) that galactose was not found in the urine of goats after parturition when the glands had been removed

A new method of experimental procedure was devised by Kaufmann and Magne (9) to determine the extent of the transformation of glucose of the blood into lactose by the mammary gland. Samples of blood were taken simultaneously from the jugular vein and the subcutaneous abdominal vein of the cow with as little disturbance as possible. They found that the glucose content of the two samples was practically the same in dry cows. In a cow about to calve, the use of glucose by the mammary glands was indicated by the lowering of the glucose content of the mammary blood over 20 per cent in comparison with the jugular blood.

In lactating cows there was considerable variation in the glucose content of the two samples of blood from the two veins—from 7 to 29 per cent—with the average of ten samples showing a decrease of 16.8 per cent. These results give added support to the theory that the glucose of the blood is the precursor of lactose.

Blackwood and Stirling (10) used the Kaufmann-Magne technique but made a correction for the concentration of the jugular blood by the withdrawal of water from the arterial blood during salivation, and found that the blood from the mammary vein was still considerably lower than that of the jugular vein.

Storage of glycogen in the mammary gland of the guinea pig is very limited according to the observations of Barrenscheen and Alders (11). On a dry weight basis they found from 1.5 to 2.0 mg. of glycogen per gram of dried residue. No significant difference was noted between the dry and lactating glands.

As regards the actual mode by which the milk sugar is formed very little is known. Since lactose occurs only in milk, it is assumed to be a synthetic product of the mammary gland. The theory has been advanced that there should be a lactose synthesizing enzyme in the mammary gland capable of converting glucose into galactose and then of combining the two hexoses into lactose. It has been also thought that there were stereokinases in the mammary gland which could convert sucrose into lactose. However, Nitzecu (12) observed that neither sucrose nor lactose could be utilized by the lactating mammary

gland Maltose, however, appeared to be utilized Hesse (13) prepared a maltosazone from a digest of macerated glands, and he believed that maltose may be an intermediary product of lactose formation Bradley (14) using gland preparations from animals in the height of lactation was unable to cause any destruction of lactose solutions of known strength and concluded that the enzyme lactase is not present and that the lactose of the milk is not synthesized through the agency of this enzyme Kleiner and Tauber (15) were unable to obtain any evidence of lactase or of lactose-synthesizing power in their glycerol udder extracts They did find a maltose enzyme in the gland extract which was capable of hydrolyzing maltose into glucose However, their animals were slaughter-house animals in an unknown condition of lactation, and furthermore, the lactose-synthesizing enzyme, if there be such in the gland, may be glycerol-insoluble

The present work which adopts the modern delicate and precise technique for the determination of sugar in blood, demonstrates that the actively lactating mammary gland does have the power of synthesizing lactose, and that the agency by which this synthesis is accomplished is not by way of a lactose enzyme which is capable of splitting lactose

EXPERIMENTAL

These preliminary experiments were undertaken in an attempt to ascertain whether the actively secreting mammary gland when dissected free from the animal, taking care not to alter any of the *in situ* conditions, would yield a synthesis of milk sugar in the test tube This would be unquestionable proof that the mammary gland itself is the site of formation of milk sugar, and not merely a secreting gland for lactose

For its convenience and availability the rat has been used as the experimental animal The rat's mammary glands, though small, are easy to dissect out, and the rat glands are apparently quite active in secreting lactose The lactose content of rat milk is reasonably high in the scale, being approximately 3.4 per cent as determined by Mayer (16) The rat nurses its young, of which it has many, for a period of 3 to 4 weeks, reaching the height of lactation at 7 to 10 days, at which time the glands were dissected out for use

The technique for removing the glands and preparing for use was as follows. The rats were anesthetized with ether. A nose bag made from a Gooch crucible in which a pluglet of cotton is inserted is very convenient for maintaining the animals under the anesthesia while operating. A median longitudinal incision was made and the skin over the two caudal glands reflected. The glands were then quickly frozen *in situ* with either a spray of ethyl chloride or by using powdered CO₂ snow packed around the gland. After the glands were frozen they were quickly dissected entirely out and placed in a dish containing powdered CO₂ snow. The glands were then placed in a high vacuum desiccator and dried, the gland remaining frozen during the entire drying process, thereby giving no occasion for autolysis.

During the drying it was occasionally necessary to refreeze the gland. The drying process took from 5 to 18 hours depending on the size of the glands. After drying was complete the gland was ground in a mortar and thoroughly mixed. The dry gland has a "moist" consistency because of the contained fat.

The initial experiment consisted of adding portions of the dried mammary gland of the rat to freshly prepared pigs' serum, incubating for a period of 12 to 24 hours, and determining the sugar content in terms of glucose by means of the copper reduction method for the determination of glucose in blood and serum of Somogyi, Shaffer, Hartmann (17). Tubes were prepared and incubated containing (1) pigs' serum plus 100 mg per cent glucose plus gland, (2) pigs' serum plus 100 mg per cent galactose plus gland, (3) pigs' serum plus 100 mg per cent lactose plus gland, and controls for the above: (4) pigs' serum plus 100 mg per cent glucose, (5) pigs' serum plus 100 mg per cent galactose, (6) pigs' serum plus 100 mg per cent lactose, (7) pigs' serum plus gland, (8) gland alone, (9) serum alone.

The amount of gland used was 0.4 gm per 5 cc. of substrate. The tubes were incubated for 18 hours at 37.5°C. after which the proteins were removed by the zinc method of Somogyi (17). It should be borne in mind that it is very important that *all* the proteins be removed from the filtrates before the sugar determinations are run. In the case of the filtrate which has only the gland plus the distilled water, the small amounts of protein which go in solution *must* be removed.

After incubation and removal of proteins (preparation of protein free filtrates) the sugar content in terms of milligrams of glucose per 100 cc. of solution was determined. Then 5 cc. samples of the filtrates were taken and fermented with washed bakers' yeast, using 1 cc. of a 10 per cent solution of yeast in water, and the reducing power again determined. It is very important that the yeast be thoroughly washed before using, by suspending in distilled water and centrifuging, resuspending and recentrifuging, repeating this process two or three times.

The results of this experiment are shown in Table I. The results are striking in that after incubation the solutions containing the active mammary gland show a significant increase in reducing power.

Furthermore, those solutions which contained the gland now have a reducing sugar which is *not* fermentable

TABLE I

In Vitro Changes Brought about by Active Mammary Gland Preparations on a Substrate of Pig Serum (Values in Terms of Glucose Per 100 Cc)

	Serum + 100 mg glucose + gland	Serum + 100 mg galactose + gland	Serum + 100 mg lactose + gland	Controls					Serum
				Serum + 100 mg glucose	Serum + 100 mg galactose	Serum + 100 mg lactose	Serum + gland	Gland + water	
After 18 hrs incubation	299	200	245	198	170	145	132	25	96
Control	233	195	170	196	172	142	121		
Increase over control	76	5	75				11		
After yeast fermentation	282	193	240	0	82	51	104	25	0
Control	25	107	76	0	76	46	25		
Increase over control	257	86	164				79		

TABLE II

In Vitro Changes Brought about by Active Mammary Gland Preparations on a Substrate of Pure Glucose (Values in Terms of Glucose Per 100 Cc)

	Glucose + gland	Controls	
		Gland + water	Glucose
After 24 hrs incubation	294	178	97
Control	275		
Increase over control	19		
After yeast fermentation	207	130	0
Control	130		
Increase over control	77		
After hydrolysis	465	279	97
Calculated lactose	187	111	0

The following experiment was made in an attempt to ascertain whether we could obtain an apparent synthesis of lactose using the entire gland in a glucose solution alone. The following tubes were prepared (1) glucose plus gland, and

controls for the above (2) distilled water plus gland, (3) glucose solution alone 0.4 gm of gland in 5 cc of substrate was used. The glucose was 100 mg per cent.

The tubes were incubated at 37.5°C for 24 hours, after which the proteins were removed as previously by the zinc method of Somogyi, and the amount of sugar in terms of milligrams per cent glucose determined. 5 cc samples of the filtrates were then fermented with ordinary yeast and the amount of sugar again determined. The sugar was determined by the recent copper iodometric method of Shaffer and Somogyi. Reagent 50 containing 5 gm of potassium iodide (18).

5 cc samples of the filtrates were then hydrolyzed by adding $\frac{1}{2}$ cc of 10 N H_2SO_4 (giving an approximately 1 N solution) and placing in a boiling water bath for a period of 2 hours. The filtrates were then neutralized with sodium hydroxide using Congo red as an indicator. It is necessary to use an indicator which has an end point slightly on the acid side as a too alkaline solution will alter the conditions of sugar oxidation. The results of this experiment are given in Table II.

It will be observed that the lactose calculated from the sugar determinations before and after hydrolysis is in agreement with the lactose as obtained by the sugar determinations before and after fermentation. The manner in which the lactose is calculated from the sugar determinations before and after hydrolysis is as follows. The reducing power of lactose is 46 per cent of that of glucose (per equal weight) as determined at this laboratory by the Shaffer-Somogyi method, Reagent 50, 5 gm KI. For galactose it is 76 per cent. If in a solution containing both glucose and lactose, A represents the total reduction in terms of glucose, X represents the glucose alone, and Y , the lactose alone in terms of glucose, then

$$X + Y = A$$

Let Z represent glucose and galactose in terms of glucose, obtained from the hydrolysis of the lactose, and B represent the total reduction after hydrolysis, then

$$X + Z = B$$

Now 100 mg of lactose (one molecule of water of hydration) on hydrolysis will yield 50 mg of glucose plus 50 mg of galactose. However, 100 mg of lactose is equivalent in reducing power to but 46 mg of glucose, and 50 mg of galactose is equivalent to $50 \times 76 = 38$ mg of glucose, so that before hydrolysis the 100 mg of lactose would be determined as 46 mg glucose and after hydrolysis be determined as $50 + 38 = 88$ mg of glucose. If Y is the number of milligrams of

lactose in terms of glucose before hydrolysis, and Z is the number of milligrams of glucose plus galactose in terms of glucose after hydrolysis, then $Z = 88/46$ $Y = 1.913 Y$ We now have two simultaneous equa-

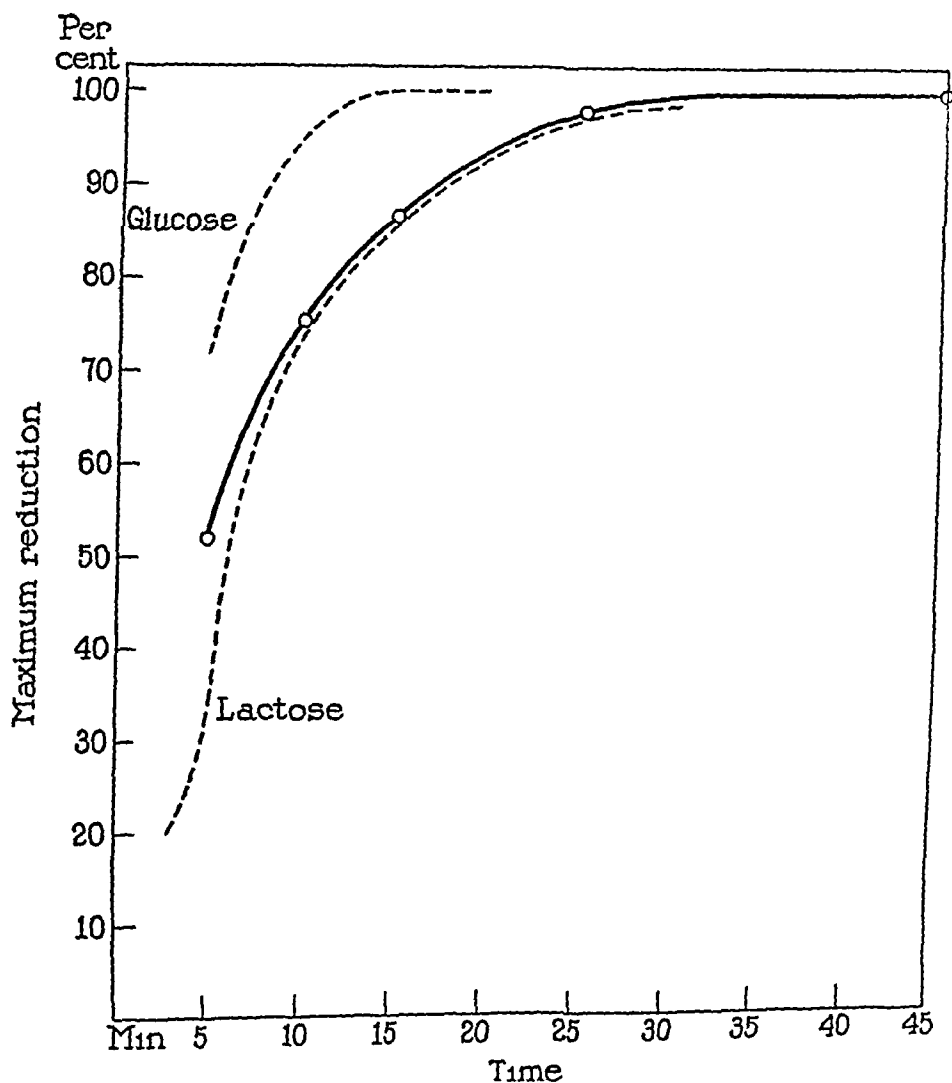


FIG 1 Velocity of reduction 0.4 gm mammary gland + 5 cc 100 mg per cent glucose

tions and two unknowns, X , the amount of glucose present, and Y , the amount of lactose present before hydrolysis

$$\begin{aligned} X + Y &= A \\ X + 1.913Y &= B \end{aligned}$$

For example, if the reducing power in terms of glucose before hydrolysis, A , equals 294, and after hydrolysis, B equals 465, then

$$\begin{array}{rcl}
 Y + X & = & 294 \\
 X \quad 19131 & = & 465 \\
 \hline
 09131 & = & 171 \\
 Y & = & 187 \text{ mg lactose in terms of glucose} \\
 & = & 187/0.46 = 407 \text{ mg lactose} \\
 \lambda & = & 294 - 187 = 107 \text{ mg glucose}
 \end{array}$$

To further corroborate the presence of lactose as a synthetic product of the active mammary gland in a pure glucose solution, the velocity of reduction of the filtrates was determined. This method for the detection of sugars in pure solution has recently been published by Shaffer and Somogyi (18). The velocity of reduction was determined for tube 1 in the preceding experiment containing 0.4 gm of rat mammary gland and 100 mg per cent glucose. The velocity of reduction as determined is given in the curve, Fig. 1.

A reproduction of the curves obtained for glucose and lactose by Somogyi is also shown in Fig. 1. By comparing the two curves it is seen that the sugar obtained from the action of mammary gland on glucose approximates the curve given for lactose, the variation during the first few minutes of boiling being due to the contained glucose.

DISCUSSION

It is at once apparent from a study of the results shown in the tables that there is a storage of some form of precursor of lactose in the mammary gland, this precursor being non-reducing but which becomes liberated and reducing when the tissue is placed in a glucose, and to some extent a lactose or galactose, solution. From Table I we see that an incubation of the active gland plus glucose plus serum gives a total reduction of 299, with the control giving 223. There is then an increase in the gland mixture of 76 mg over the control, this increase necessarily coming from the gland tissue. Galactose apparently inhibits the liberation or formation of lactose from the stored material to some extent, while lactose has also a slight inhibitory effect.

In the case of the filtrate containing the glucose, serum, and gland, it is seen that the glucose of the serum and the added glucose were converted almost quantitatively into lactose, 299 before against 282

after fermentation This means a conversion of 179 mg of glucose into lactose giving after conversion a reduction of but $0.46 \times 179 = 82$ The remaining constituent is the gland alone and has a reduction value of 25, giving a total calculated reduction in the mixture after fermentation of $82 + 25 = 107$ The amount liberated from the gland would then be $282 - 107 = 175$ mg (determined as glucose)

Comparing this with the filtrate containing the lactose, serum, and gland, it is seen that the glucose of the serum was also converted almost quantitatively into lactose, 245 before against 240 after fermentation This means a conversion of 91 mg of glucose in the serum to lactose, giving after conversion a reduction of but $0.46 \times 91 = 42$ The remaining constituents, gland plus lactose, have a reducing value of 25 and 46 respectively, the total being $41 + 25 + 46 = 112$ The amount liberated from the gland is then $240 - 112 = 128$

In the case of the filtrate containing the galactose, the glucose of the serum (96 mg) was again converted almost quantitatively into lactose, 200 before against 193 after fermentation If we again assume that this 89 mg of glucose is actually converted into lactose, it would then have a reducing value of $0.46 \times 89 = 41$ The remaining constituents, gland and galactose, have a reducing value of 25 and 76 respectively, the total being $41 + 25 + 76 = 142$ The amount liberated from the gland is then $193 - 142 = 51$

The glucose-containing filtrate effected a liberation from the gland of 175 mg of lactose (determined as glucose), the lactose filtrate effected a liberation of 128, and the galactose filtrate effected a liberation of but 51 mg of lactose

In the case of the second rat (Table II) the results are not so striking as the first animal Here we have the values 294 before against 207 after fermentation Since we added to the gland 97 mg of glucose, and we have in the gland $178 - 130 = 48$ mg of fermentable reducing substance, we may consider that we have a lactose-forming potentiality here of $97 + 48 = 145$ mg However, only a part of this was converted to lactose, $294 - 207 = 87$ mg was fermented out This leaves $145 - 87 = 58$ mg of glucose converted to lactose and then giving a reduction value of $0.46 \times 58 = 27$ The amount of precursor liberated (as lactose) would then be $207 - (178 + 27) = 2$ or practically nothing

Here then, we did get a synthesis of lactose from added glucose, with no concurrent liberation of lactose precursor as in the preceding experiment

The explanation of this lies probably in the fact that this animal was taken just prior to being suckled, the mammary glands being engorged with contained milk, while the previous animal was taken after being suckled, the glands being practically devoid of milk. This would indicate that in the case of the second rat the lactose precursor storage depots have been exhausted, and will not be replenished until the gland is emptied, while in the first animal the gland was caught at the height of its lactose precursor storage activity in preparation to filling the gland with milk.

The indications are then that there is a rhythm of activity of the gland depending on the cyclic emptying of the gland of secreted milk. This rhythm is manifested by a storage in the gland itself of some substance which is readily and rapidly converted to lactose when the call is made for it, that is, when the gland is emptied of contained lactose—by suckling or milking—there is a liberation and conversion of this substance into lactose, then a subsequent filling and engorgement of the tubules of the gland with milk, and again a storage of milk precursor. It is a well known fact that failure to milk a lactating animal will cause a "drying up," while continued milking causes continuous secretion.

The place of synthesis of lactose is unquestionably in the mammary gland. The second hypothesis of Bert, that the lactose was formed in the blood and secreted in the mammary gland, could not be true. However, his first hypothesis, that the lactose was formed by constituents stored in the mammary gland, similar to the storage of glycogen by the liver, may be partially true. There is a storage of lactose precursor in the gland, which, however, seems to require a substrate of sugar to be liberated. In other words, the blood with its contained sugar is necessary for the formation of milk sugar, and not only is the glucose of the blood the prime precursor of the milk sugar but it is a mechanism for the liberation and formation of the lactose from its precursor.

That the reducing non fermentable disaccharide which is being obtained as a synthetic product of lactating mammary gland action is

lactose seems to be indirectly proven. When the gland is placed in a solution of glucose and incubated, the glucose is used up, and some non-fermentable substance appears in its place.

This non-fermentable substance is reducing, and is demonstrated to be a disaccharide by the hydrolysis experiments. Further, it is shown to be lactose by its specific velocity of reduction of an alkaline copper solution. Since the filtrate used in determining the velocity of reduction had some glucose in it, the curve obtained shows an initial velocity of reduction higher than for pure lactose, but since the glucose reaches its maximum reduction in 5 minutes the curve obtained approaches more nearly that for pure lactose.

SUMMARY

1 The mammary glands of rats in the height of lactation were frozen *in situ*, removed, and dried in the vacuum in the frozen state.

2 The dried gland, added to pig serum, pig serum plus glucose, pig serum plus galactose, and pig serum plus lactose, then incubated at 37.5°C, yielded a synthesis of non-fermentable, reducing material.

3 The dried gland added to glucose solution alone and incubated at 37.5°C yielded a synthesis of a non-fermentable reducing disaccharide, having a reaction velocity of reduction resembling that of pure lactose.

4 There is a "short time" storage of some forms of lactose precursor in the active mammary gland. This precursor is non-reducing but becomes available and reducing when the gland is placed in a solution containing glucose.

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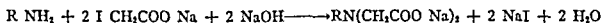
THE KINETICS OF THE REACTION WHICH TAKES PLACE BETWEEN IODOACETIC ACID AND GLYCINE*

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In a recent communication, Michaelis and Schubert (1) called attention to the fact that iodoacetic acid reacts quite readily with the amino group according to the equation,



Since a similar but relatively faster reaction also takes place between the SH group and iodoacetic acid which probably accounts for the inhibitory action of iodoacetic acid on certain physiological processes (2), the question arises whether the rate of the reaction between the amino group and iodoacetic acid is sufficiently rapid over the physiological pH range to have some practical significance

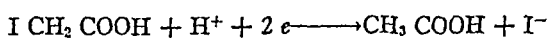
In order to decide this question, the velocity constants of the reaction between the amino group and iodoacetic acid must be known. Since the pH of the medium in which the reaction takes place influences the rate considerably, buffer solutions having a large reserve must be used. This involves the difficulty of following the change in concentrations of the reactants with time by ordinary analytical methods. An attempt to apply Warburg's manometric method for the determination of the reaction rate between halogen acetates and the SH group was made by Dickens (2). He assumed that the amount of CO_2 which is produced when the reaction takes place in a CO_2 -bicarbonate buffer solution is equivalent to the amount of HI which is liberated during the reaction and, therefore, to the decrease of the reacting components. In such buffer solutions, however, carbamates

*Aided by a grant from The Chemical Foundation Inc., and the Research Board of the University to Professor Carl L. A. Schmidt

†International Education Board Fellow

are formed from amino acids (3) A considerable influence as a result of this factor was observed when the reaction between alanine or glycine and iodoacetic acid was followed in a sodium carbonate buffer solution (4) The use of borate or phosphate buffers eliminates the manometric method for the estimation of reaction rates

The polarographic technique which has been developed by Heyrovský (5) was employed for the study of the present problem It is a special electrolytic method which employs a mercury dropping electrode as the cathode and a mercury electrode of the second order as the anode The course of the electrolysis is recorded automatically on photographic paper as current voltage curves On the basis of these curves, both the qualitative and the quantitative composition of the solution under investigation can be determined When several electro-reducible compounds are present in the solution, they can be distinguished from each other according to the differences in their characteristic reduction potentials, and their individual concentrations can be estimated from the values of the limiting current intensities which correspond to the horizontal portions of the curve The current-voltage curves of the solutions which contain iodoacetic acid show a distinct reduction which corresponds most probably to the reaction,



The limiting current intensities of this reaction measured in millimeters (see Fig 1) are strictly proportional to the concentration of iodoacetic acid The proportionality constants expressed by the ratio

$\frac{\text{Limiting current}}{\text{Concentration of iodoacetic acid}}$ are given in Table I Quantitative estimations of iodoacetic acid in buffer solutions can be carried out in 2 minutes with an accuracy of ± 1 per cent

EXPERIMENTAL

The current-voltage curves were recorded by means of a Type VII polarograph¹ The sensitivity of the galvanometer was 1.2×10^{-9} amp/mm, and its half-time period was 4.53 seconds The sensitivity could be decreased by means of a special shunt The present measurements were made at $\frac{1}{200}$ or $\frac{1}{500}$ of the above sensi-

¹Manufactured by Drs V and J Nejedlý, Prague, Czechoslovakia

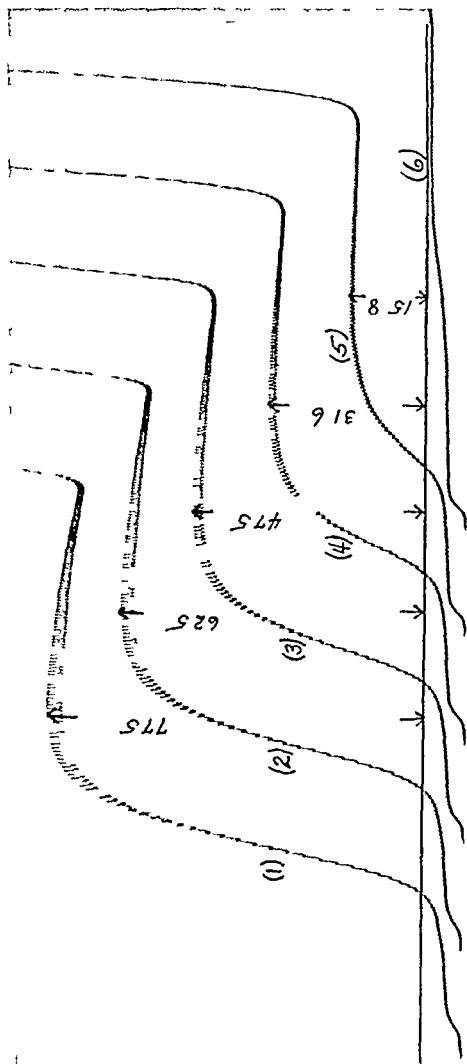


Fig 1 Current voltage curves (sensitivity of galvanometer I_{μ})

No

1 $(9.92 \times 10^{-3} \text{ N})$

2 $(1.92 \times 10^{-3} \text{ N})$

3 $(5.92 \times 10^{-3} \text{ N})$ sodium iodoacetate in 0.08 N sodium carbonate solution

4 $(3.96 \times 10^{-3} \text{ N})$

5 $(2.00 \times 10^{-3} \text{ N})$

6 Atmospheric oxygen dissolved in 0.08 N sodium carbonate solution

tivity value This is most convenient for the $4 \times 10^{-3} - 4 \times 10^{-4}$ equivalent concentration of iodoacetic acid range to which the solutions were diluted

The glycine and iodoacetic acid were checked as to their purity The usual standardization of the hydrogen electrode against buffer mixtures was carried out The sodium salt of iodoacetic acid and glycine in a sodium borate buffer solution were used chiefly for the rate measurements at different pH values In order to determine the reaction rates when glycine in a borate buffer solution is treated with iodoacetic acid, it is necessary that the pH values of the solutions of the buffer components be known The titration curves are shown in Fig 2

The following procedure, in general, was employed for the rate determination to a known volume of the glycine-containing buffer mixture, a certain amount of 0.5 N sodium iodoacetate was added The mixture was incubated at $25^\circ \pm 0.01^\circ$ 5 cc portions were withdrawn at certain time intervals and added to an amount of HCl which brought the mixture approximately to neutrality in order to stop the reaction Since the iodide ion, which is one of the products of the reaction, affects the potential of the anode, giving to it a more negative value, the HCl was mixed

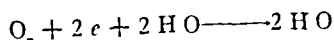
TABLE I

Curve	1	2	3	4	5
Limiting current (mm on scale)	77.5	62.5	47.5	31.6	15.8
Concentration of iodoacetic acid (equiv's $\times 10^3$)	9.92	7.92	5.95	3.96	2.0
Ratio	7813	7891	7983	7980	7900

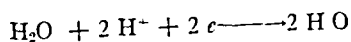
with 0.04 N KI in order to maintain constant conditions at the anode during the polarographic estimations

A mercury cathode dropping at a 3 seconds rate was used The electromotive polarizing force was supplied from a 4 volt lead battery The vertical lines on the polarograms (Figs 1 and 3) correspond to 200 mv It was necessary to record only a part of the horizontal portions of the current-voltage curves From these curves the concentrations of iodoacetate in the solutions were determined The horizontal portions were obtained by using a polarizing E.M.F. of 1.16 volts (see Fig 3)

Besides the reduction of the iodoacetate, the current-voltage curves include the reduction of the atmospheric oxygen which is present in the aqueous solutions in about 10^{-3} equivalent concentration This reduction takes place in 2 steps (Fig 1, Curve 6) the first corresponding to the reaction



and the second to



It is possible to remove the oxygen by blowing hydrogen or nitrogen through the solution. This procedure was not followed since the current due to the oxygen reduction can be determined in the buffer solu-

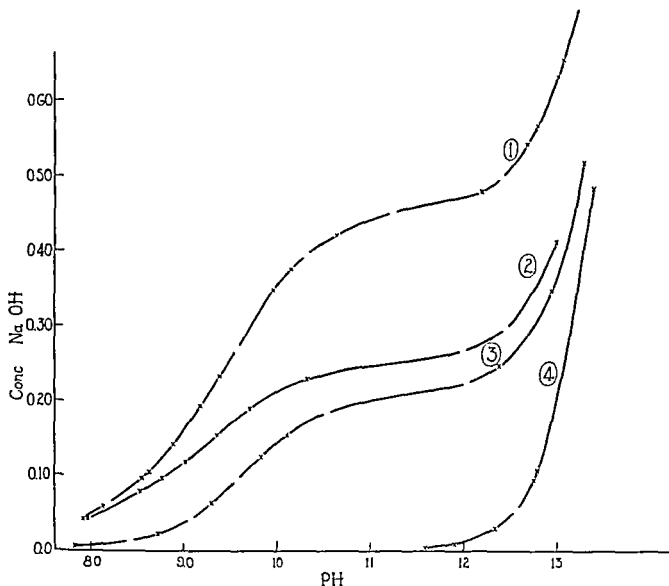


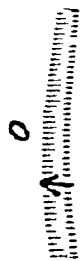
FIG. 2 Electrometric titration curves

No

- 1 0.204 N glycine + 0.247 N boric acid in 0.1 N KCl
- 2 0.247 N boric acid in 0.1 N KCl
- 3 0.204 N glycine
- 4 Water blank

tions and subtracted from that of the corresponding solutions which contained iodoacetate. The horizontal line recorded on each set of estimations (Fig. 3) accounts for the current intensity due to the reduction of oxygen so that the distance between this and the horizontal

(a)



30



63

47

6.3

340

200

35

23.5

35

26.5

150

90

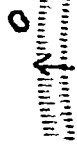
60

42.8

30

0

(b)



30

60

90

60

52

63

200

35

340

Fig. 3. Limiting current intensities due to the electro reduction of iodoacetates. These are proportional to the values of $(a-X)$, recorded at different times t (in minutes) during the course of the reaction of iodoacetate with glycine in a borate buffer solution (a) at pH 9.76 (decreasing curves from left to right), (b) at pH 9.46 (decreasing curves from right to left) (Sensitivity of galvanometer 10^{-10})

portion of the iodoacetate current voltage curve corresponds to its actual concentration

In studying the reaction which takes place between glycine and iodoacetic acid in an alkaline buffer solution, consideration must be given to the fact that a single reaction does not take place. Iodoacetic acid is saponified by hydroxyl ions yielding glycollic acid (6). The total decrease in concentration of iodoacetate which is observed experimentally therefore involves two independent reactions. Allowance was made for the effect of the saponification reaction by parallel experiments in which the glycine was eliminated. The determination of the velocity constants of the saponification reaction will be reported in another paper (7).

Since there is an ample reserve of OH^- ions in the buffer mixtures, the rate of saponification of iodoacetic acid can be expressed by an equation of the first order in which the monomolecular velocity constant, k_s , is related to pH.

When the saponification takes place simultaneously with the glycine iodoacetic acid reaction, the equation for the saponification must be written in the form,

$$\frac{d}{dt} = k(a - X - z) \quad (1)$$

where a is the initial equivalent concentration of the iodoacetic acid, z its decrease due to saponification during the time t , and X , the decrease of iodoacetic acid resulting from its reaction with glycine.

Even when the value for X as a function of t is not known, the value for z can be obtained from the value for k_s which was determined in the study of the saponification reaction, and the value for $(a - X - z)$, which denotes the estimated concentration of iodoacetic acid at time t in the glycine iodoacetate mixture. If the values for $(a - X - z)$ are plotted against t , then for any value of t the area below the plotted curve, multiplied by the value for k_s , determines the value for z (see Table II and Fig. 4). Knowing the value for z , it is possible to calculate values for $(a - X)$. The curve which is obtained by plotting $(a - X)$ against t (Fig. 4) shows the rate of decrease of iodoacetate as a result of its reaction with glycine.

It would be expected that the reaction rate between glycine and iodo

acetic acid should be trimolecular. The experimental data, however, do not bear this out. They conform more closely to the bimolecular type of reaction. The explanation that only one hydrogen of the amino group is replaced is not correct, since chemical findings show that the disubstituted product is formed (1). These views can be reconciled by the assumption that the reaction rate is only apparently bimolecular, the kinetics being different from that which would be expected from the chemical equation

TABLE II

	$a-X-z$	$\int_0^t (a-X-z)dt$	z	λ	$\frac{d\lambda}{dt}$	$\frac{1}{a-X} \frac{d\lambda}{dt}$	$\frac{k_1'}{2} (2c-\lambda)^*$
0	56.8	0	0	0	0.244	0.00429	0.00429
50	46	2570	0.95	9.8	0.162	0.0034	0.0032
180	32	7545	2.8	22	0.070	0.0020	0.0018
270	26.1	10160	3.8	26.9	0.042	0.0014	0.0012
360	22.6	12351	4.6	29.6	0.028	0.0010	0.0009
480	19.1	14853	5.5	32.2	0.019	0.0007	0.0006
800	14.8	20245	7.5	34.5			
1200	11	25405	9.4	36.4			

$$a = 0.10 \text{ N}$$

$$c = 0.033 \text{ N}$$

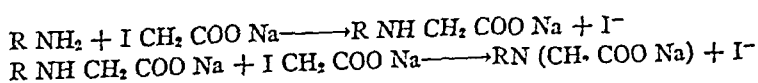
$$\text{pH} = 12.96$$

$$k_s = 3.7 \times 10^{-4}$$

$$k_1' = \left(\frac{dX}{dt} \right)_0 \frac{1}{ac} = 0.130$$

* Since X is expressed in arbitrary units, its value has to be transformed into equivalent concentration. If the concentration of a ($a = 0.01 \text{ N}$) corresponds to 56.8 mm units, then in order to express X in equivalent concentrations, it has to be multiplied by the factor, $\frac{0.1}{56.8}$

There are many examples in chemical kinetics which show that two or more substitutions in the same molecule usually proceed in steps and that each substitution reaction possesses its own velocity constant (8). If this be the case when iodoacetic acid and glycine react, the following reactions must take place



Since both of these reactions are bimolecular, the following kinetic equations express the relations

$$\frac{dx_1}{dt} = k_1(a - x_1 - x_2)(c - x_1) \quad (2)$$

$$\frac{dx_2}{dt} = k_2'(a - x_1 - x_2)(x_1 - x_2) \quad (3)$$

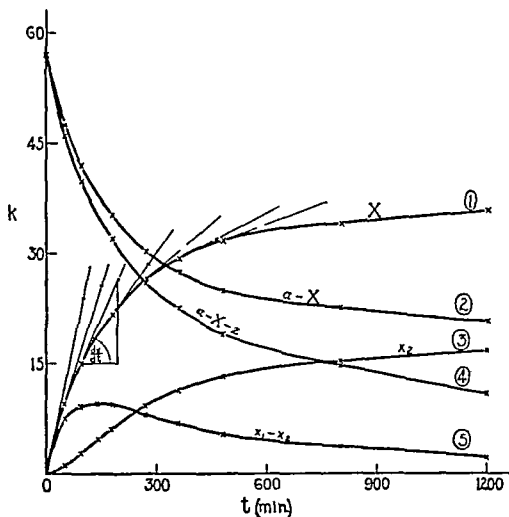


FIG. 4. Curves showing the changes of $(a - Y - x)$, $(a - X)$, λ , $(x_1 - x_2)$ and x_2 , with time during the reaction between 0.01 N sodium iodoacetate and 0.033 N glycine in a borate buffer of pH 12.96. The ordinates are represented in arbitrary units which are proportional to the equivalent concentrations of the reacting components.

where a and c represent the initial equivalent concentrations of iodoacetate and glycine, respectively, x_1 , the total decrease in the concentration of glycine at the time t , and x_2 , the decrease in the concentration

of monosubstituted glycine which is equal to the concentration of the disubstituted product

The concentration of iodoacetate at any time is equal to $(a - x_1 - x_2)$, that of glycine to $(c - x_1)$, and that of the monosubstituted form to $(x_1 - x_2)$. The apparent velocity constants for each step in the reaction at the given pH are designated by k'_1 and k'_2 .

In general, these differential equations cannot be solved for k'_1 and k'_2 . However, by eliminating t , the following relationship can be obtained

$$\frac{dx_1}{dx_2} = \frac{k'_1}{k'_2} \frac{(c - x_1)}{(x_1 - x_2)} \quad (4)$$

which, when solved, gives

$$k'_1 c - k'_2 (x_1 - x_2) - k'_1 x_2 = k'_1 c \left(1 - \frac{x_1}{c}\right)^{\frac{k'_2}{k'_1}} \quad (4a)$$

(when $x_1 = 0$, it is assumed that $x_2 = 0$)

The sum $(x_1 + x_2)$ only can be calculated from the experimental values of the concentration of iodoacetate at certain time intervals. This value is identical with the previously mentioned value for X .

The addition of equations (2) and (3) gives

$$\frac{dX}{dt} \frac{1}{a - X} = k'_1 c - x_1(k'_1 - k'_2) - k'_1 x_2 \quad (5)$$

By combining equations (5) and (4a), a further relationship is obtained

$$\frac{1}{a - X} \frac{dX}{dt} = k'_1 (2c - X) - k'_1 c \left(1 - \frac{x_1}{c}\right)^{\frac{k'_2}{k'_1}} \quad (5a)$$

Values for the left side of this equation can be determined experimentally as $\frac{dX}{dt}$ represents the tangent to the curve when X is plotted against t (see Fig. 4). This tangent for zero time when $X = 0$ determines the value for k'_1 according to the relationship

$$\frac{dX}{dt} = a k'_1 c \quad (5b)$$

The experimental values for other tangents show that the following relationship is satisfied (see Table II)

$$\frac{1}{a - \lambda} \frac{d\lambda}{dt} = \frac{k_1}{2} (2c - \lambda) \quad (6)$$

From equation (5a) the following relationship must also hold

$$\frac{k_1}{2} (2c - \lambda) = k_1 c \left(1 - \frac{x_1}{c} \right)^{\frac{k_1}{k_1}} \quad (6a)$$

If the right side of this equation is replaced by the left side of equation (4a), equation (6a) assumes the form

$$\frac{k_1}{2} (2c - \lambda) = k_1 c - k_2 (x_1 - x_2) - k_1 x_2 \quad (6b)$$

Both sides of this equation will be identical in only one case, viz, when $k'_1 = 2 k'_2$. Using this relationship in equations (2) and (3), substituting λ for $(x_1 + x_2)$, and integrating, the following equation is obtained

$$k_1 = \frac{2}{1(2c - a)} \ln \frac{a}{2c} \frac{(2c - \lambda)}{(a - \lambda)} \quad (7)$$

This is similar to the equation for the bimolecular reaction

It has been pointed out by Abel (9) that some reactions which show a lower order of rate than would be expected from the chemical equation can be explained as consecutive reactions when the ratio, $1/2 \rightarrow$, is assumed for the velocity constants. He interprets (10) the results of Kremann dealing with the saponification of glycodiacetate, which show that the course of the reaction is apparently bimolecular instead of trimolecular, on this basis (11). If the steric effect is disregarded, the physical basis for the simple ratio of the velocity constants accounts for the probability of an effective collision. Thus, for example, a molecule in which equivalent substitutions can take place will have a twofold greater chance for such a collision than when one group is already substituted. The correctness of this principle was confirmed in the investigations on the hydrolysis of polybasic esters (12).

Disregarding the secondary effects which will be discussed later, the consecutive character of the glycine iodoacetate reaction having

velocity constants in the ratio 2 : 1 appears to be supported amply by the present experiments. The ratio of the velocity constants involves in the first place a solution of the relationships between the concentrations of the mono- and the disubstituted form of glycine when the total decrease X of iodoacetate has been determined experimentally.

These relationships follow from equation (4a) under the condition when $k'_1/k'_2 = 2 : 1$, and are expressed by the following equations

$$x_1 = X - \frac{X^2}{4c} \quad (8)$$

$$(v_1 - x_2) = X - \frac{X^2}{2c} \quad (9)$$

$$x_2 = \frac{X^2}{4c} \quad (10)$$

The relationship between t and the components is represented graphically in Fig. 4 for the case $a = 0.01 \text{ N}$, $c = 0.033 \text{ N}$, and $\text{pH} = 12.96$.

According to equation (9), if $a > c$, then the concentration of monosubstituted glycine ($v_1 - x_2$) will reach a maximum when $X = c$. The corresponding time can be expressed by equation (7) in which $X = c$.

$$t_{\max} = \frac{2}{k'_1(a - 2c)} \ln 2 \left(1 - \frac{c}{a} \right) \quad (11)$$

For the same time, x_2 shows an inflection point. The monosubstituted glycine will disappear entirely at the end of the reaction only in the case in which $a \geq 2c$, i.e. when the initial concentrations are in accordance with the stoichiometric chemical equation or, if the amount of iodoacetic acid is in excess of the stoichiometric ratio. In every other case a mixture of the mono- and disubstituted form will be obtained, the ratio of which is given by equations (9) and (10). When $c > a$, the concentration of the monosubstituted form will not reach a maximum. The yield of the mono- will be greater than the yield of the disubstituted form.

It is more convenient for purposes of further study to assume the condition when the initial concentration of c is greater than that of a in order to show the relationship between the velocity constants and

pH The buffering action of glycine itself helps to maintain in part the constancy of the pH during the reaction and, in part, to increase the reaction rate. If an excess of glycine over iodoacetate is used such that the reaction rate can be treated as being approximately monomolecular, the calculated value for the velocity constant is in reality the sum of k_1c and k (velocity constant of saponification). Experience shows (7) that k_1 , being of the order of 10^{-4} — 10^{-6} over the pH range investigated, can be neglected when the initial concentrations of glycine and iodoacetate respectively are about 0.2 and 0.02 N.

TABLE III

(a) pH = 9.76				(b) pH = 9.46		
Time	(a-X)	(2c-X)	$k_1 \times 10^3$	(a-X)	(2c-X)	$k_1 \times 10^3$
min						
0	63	1305		63	1305	
30	47	1289	4.80	52	1294	3.14
60	35	1277	4.84	42.8	1284	3.17
90	26.3	1270	4.82	35.3	1277	3.28
150	15.3	1257	4.71	23.5	1265	3.26
200	9.5	1251	4.75	16.7	1258	3.31
340	2.5	1244	4.80	6.3	1248	3.34

* (2c-X) must be expressed in the same arbitrary units as (a-X). Since for $a = 0.0198$ N the value in the arbitrary units equals 63, for c the value of 0.205 $\times \frac{63}{0.0198} = 1305$ must be used. It is immaterial which units in the term behind the logarithms in equation (7) are used. The term (2c-a) before the logarithms, however, must be expressed in equivalent concentrations.

Table III gives the data for the calculation of the velocity constant, k_1 , according to equation (7) for two sets of experiments which were carried out in borate buffer solutions at pH 9.76 and 9.46, respectively (see also Fig. 3). Table IV contains the average values of the velocity constant, k_1 , over the investigated range of pH, calculated with the aid of equation (7). This is the same method as was used in calculating the k_1 values which are given in Table III. The pH values of the reacting solutions for any given concentration of NaOH were estimated from the titration curves which are given in Fig. 2.

The titration curve of the glycine borate buffer mixture (Curve 1,

Fig 2) shows the pH change which must take place during the reaction between glycine and iodoacetate as a result of the neutralization of a part of the NaOH by HI. From the data which are given in Table IV, it can be seen to what extent the reaction rate depends on the pH. A decrease in the value of the velocity constants with decrease of pH during the reaction should be expected. Such an effect, however, was

TABLE IV

pH	NaOH	$k'_1 \times 10^2$	b	$\frac{(k'_1 \times 10^2)c}{b} = k_1$	μ
8.18	0.062	0.21	0.0062	6.91	0.162
8.63	0.104	0.60	0.017	7.20	0.204
8.89	0.145	1.18	0.029	8.30	0.245
9.13	0.186	1.90	0.047	8.34	0.286
9.46	0.249	3.40	0.083	8.36	0.349
9.66	0.293	4.35	0.106	8.37	0.393
9.76	0.312	4.80	0.117	8.37	0.412
9.85	0.331	5.40	0.130	8.47	0.431
9.96	0.350	5.95	0.143	8.49	0.450
9.98	0.353	6.00	0.144	8.50	0.453
10.02	0.358	6.20	0.148	8.55	0.458
10.14	0.373	6.65	0.159	8.53	0.473
10.29	0.395	7.30	0.173	8.61	0.495
10.56	0.416	8.05	0.191	8.60	0.516
11.22	0.455	9.30	0.204	9.30	0.555
11.52	0.466	9.60	0.204	9.60	0.566
12.16	0.482	10.0	0.204	10.0	0.582
12.38	0.500	10.1	0.204	10.1	0.600
12.64	0.545	10.4	0.204	10.4	0.645
12.94	0.635	10.8	0.204	10.8	0.735
13.17	0.725	11.2	0.204	11.2	0.825

Initial concentration of (a) iodoacetate, 0.0198 N, (b) glycine, 0.204 N, (c) boric acid, 0.297 N, (d) KCl, 0.1 N

Maximum deviation of $k'_1 = \pm 4$ per cent

not observed. Only one explanation remains, viz., that the decrease in the values of the velocity constants is balanced by another effect. This will be discussed in the paragraph dealing with salt effects.

If the values for the velocity constants, k'_1 , are plotted against pH, a sigmoid curve is obtained which is very similar to the titration curve of glycine (see Fig. 5). It can be shown that the velocity constants,

k'_1 , for any pH value are nearly proportional to the concentration of glycinate anion. The latter can be determined from Curve 3 of Fig. 2 at the corresponding pH. This fact indicates that the rôle played by pH in the glycine iodoacetate reaction consists in shifting the equi-

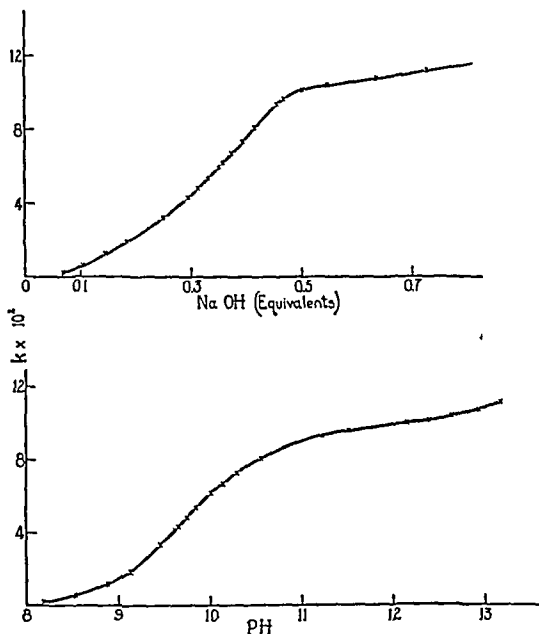
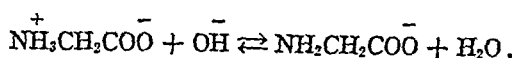


FIG. 5 The relation of the constants, k'_1 calculated on the basis of equation (7) to (a) pH and (b) the concentration of NaOH

librium between the two forms of glycine of which only the anionic form is reactive

In order to obtain the velocity constants independent of pH, the degree of dissociation of glycine has to be taken into account. In an

alkaline solution an equilibrium exists between the two forms of glycine which, in terms of the zwitter ion theory, is expressed by



and is defined by the apparent zwitter ionic constant, K'_B

Denoting the analytical concentration of glycine by c , and the concentration of its anionic form by b , the following relationship must hold

$$K'_B = \frac{(c-b)(\text{OH}^-)}{b} \quad (12)$$

If the anionic form only is able to react, the decrease in glycinate concentration, x_1 , must be balanced by the formation of anionic glycine from the zwitter ionic form in order to maintain the equilibrium. Denoting the amount of zwitter ionic glycine which is transformed into the anionic form by y , then

$$K'_B = \frac{(c-b-y)(\text{OH}^-)}{(b-x_1+y)} \quad (12a)$$

The term, $(b-x_1+y)$, must be used in equation (2) in place of $(c-x_1)$. By assuming a constant value for pH and combining equations (12) and (12a), the equation,

$$(b-x_1+y) = (c-x_1) \frac{(\text{OH}^-)}{K'_B + (\text{OH}^-)}, \quad (12b)$$

is obtained

The kinetic equation which contains the velocity constant independent of the pH assumes the form

$$\frac{dx_1}{dt} = k_1 \frac{(\text{OH}^-)}{K'_B + (\text{OH}^-)} (c-x_1)(a-x_1-x_2) \quad (2a)$$

The same reasoning can be applied to the second step of the reaction. The assumption has to be made that the same zwitter ionic equilibrium exists for the monosubstituted form of glycine as for glycine itself. This assumption appears to be affirmed by the titration curve of trimethyl carboxylamine (1). In this case a certain amount of mono-

substituted glycine which is formed from glycine anions must be converted to the zwitter ionic form according to the equilibrium,

$$K_B = \frac{y_1(\text{OH}^-)}{x_1 - y_1}, \quad (13)$$

where y_1 represents the concentration of the zwitter ionic form of monosubstituted glycine

The decrease x_2 of monosubstituted glycine in the second step of this reaction is balanced by the formation of monosubstituted glycine anion from the zwitter ionic form. Designating the amount of the zwitter ionic form thus transformed by y_2 , then

$$K_B = \frac{(y_1 - y_2)(\text{OH}^-)}{x_1 - y_1 - x_2 + y_2} \quad (13a)$$

The term, $(x_1 - x_2)$, must be replaced by $(x_1 - x_2 - y_1 + y_2)$ in equation (3). By combining equations (13) and (13a), the equation

$$(x_1 - x_2 - y_1 + y_2) = \frac{(\text{OH}^-)}{K_B + (\text{OH}^-)} (x_1 - x_2) \quad (13b)$$

is obtained

The second kinetic equation which is defined by velocity constants which are independent of pH assumes the form

$$\frac{dx_2}{dt} = k_2 \frac{(\text{OH}^-)}{K_B + (\text{OH}^-)} (x_1 - x_2)(a - x_1 - x_2) \quad (3a)$$

The integration of the sum of differential equations (2a) and (3a) gives

$$k_1 = \frac{K_B + (\text{OH}^-)}{(\text{OH}^-)} \frac{2}{t(2c - a)} \ln \frac{a}{2c} \frac{(2c - X)}{(a - X)} \quad (7a)$$

It follows from equation (12) that

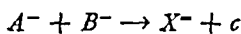
$$\frac{K_B + (\text{OH}^-)}{(\text{OH}^-)} = \frac{c}{b}$$

The value for the velocity constant, k_1 , can be obtained from any of the values for k'_1 (Table IV) by multiplying them by the value for the ratio $\frac{c}{b}$. The value for b at a given pH can be obtained from the titration curve of glycine (Curve 3, Fig. 2)

These velocity constants which have to be regarded as being independent of pH are given in the 5th column of Table IV

The conclusion that only the anionic form of glycine reacts with iodoacetate indicates that this reaction is of the ionic type. Since one of the reacting components is supplied by a weak electrolyte, both primary and secondary salt effects must take place according to Bronsted's hypothesis (13). In accordance with this hypothesis, the velocity constants which were calculated on the basis of the above equations are functions of the salt concentrations in the reacting medium.

Under the present experimental conditions, the first step of the consecutive reactions predominates. The second step may, therefore, be disregarded.² The first step corresponds to the reaction type which is given by Bronsted, *viz*



where A^- denotes the glycine anion, B^- the iodoacetate anion, and X^- an unstable "critical" complex which represents an addition compound between the two reacting ions. In such a case a positive catalytic salt effect is to be expected. It is characterized generally by the factor F which depends on the salt concentration in the reacting medium. The measured velocity constants are equal to Fk_0 where k_0 represents a constant which is independent of the salt concentration.

The factor F is determined by the activity coefficients of the reacting ions, f_{A^-} , f_{B^-} , and of the critical complex, f_{X^-}

$$F = \frac{f_{A^-} - f_{B^-}}{f_{X^-}}$$

In the extreme dilution where the Debye-Huckel limiting law for activity coefficients can be applied, the value for F can be calculated from the ionic strength μ . For the type of reaction in question, its value should be equal to $10^{\mu^{1/2}}$.

Since the ionic strengths of the solutions which have been investi-

²It can be shown by means of equations (9) and (10) that, at the end of reaction, 97.6 per cent of the glycine which reacted with iodoacetate was transformed to monosubstituted glycine, and 2.4 per cent to the disubstituted form when the initial concentrations of glycine and iodoacetate were 0.2 N and 0.02 N, respectively.

gated are greater than 0.1μ (see Table IV, Column 6), neither this limiting law nor the Bronsted expression,

$$\ln \frac{f_A f_B}{f_{X^-}} = 2\alpha\mu^{1/2} - (\beta_A + \beta_B - \beta_X)c$$

which holds for middle concentrations, can be used (α is a universal constant for all ions and β depends on their character (14))

By considering the primary salt effect only, the velocity constant, k_0 , independent of the salt concentration, can be calculated when the value for I for one ionic strength is known. In the present case, however, a further complication arises since the primary salt effect interferes with the secondary salt effect which is related to the concentration of the reacting glycine anion.

It follows from the zwitter ionic concept that the equivalent concentration of the glycine anion is given by

$$(\text{NH}_2\text{CH}_2\text{COO}^-) = \frac{f_{A^\pm}}{f_{A^-} f_H} \frac{(\text{NH}_2^+\text{CH}_2\text{COO}^-)}{(\text{H}^+)} \frac{a_w}{K_B}$$

where f_{A^-} , f_{A^\pm} , and f_H represent the activity coefficients of glycine anion, glycine zwitter ion, and hydrogen ion, respectively, a_w the ionic product of water, and K_B the true zwitter ionic constant of glycine.

By determining the velocity constants, k_1 , independent of pH (Column 5, Table IV), the concentrations of glycine anion were read from the glycine titration curve (Curve 3, Fig. 2) at the corresponding pH at which the reaction was carried out. This was not entirely correct, since, in the reacting medium where the ionic strength was always higher than in case of the solutions used for the titration curves, the activity coefficients f_{A^-} , on the other hand, possessed a smaller value. This fact influences, at the same time, the change in the equilibrium concentrations of glycine anion.

At a given pH the concentration of the glycine anion can be expressed as a function of its activity coefficient, γ :

$$\frac{(\text{NH}_2\text{CH}_2\text{COO}^-)}{c - (\text{NH}_2\text{CH}_2\text{COO}^-)} = K \frac{1}{f_{A^-}} \quad (14a)$$

or,

$$(\text{NH}_2\text{CH}_2\text{COO}^-) = \frac{Kc}{K + f_{A^-}} \quad (14b)$$

where

$$K = \frac{a_w f_{A\pm}}{K_B(H^+)f_{H^+}}$$

The value of K is approximately independent of the ionic strength since $f_{A\pm}$ is affected very little by the presence of salts (15). Since values for the thermodynamic constant, K_B and a_w are known ($K_B = 6.04 \times 10^{-5}$, $a_w = 1.01 \times 10^{-14}$) (16), the value of K can be determined for various pH values. At pH 8, $K = 1.67 \times 10^{-2}$. The value for K increases tenfold for each unit in the pH value.

When the concentration of glycine anion, $(\text{NH}_2\text{CH}_2\text{COO}^-)_1$, (obtained from the titration curve), is compared at the same pH with its concentration in the reacting medium, the following relationship is used

$$\frac{(\text{NH}_2\text{CH}_2\text{COO}^-)_1}{(\text{NH}_2\text{CH}_2\text{COO}^-)} = \frac{K + f_{A^-}}{K + (f_{A^-})_1} \quad (15)$$

where f_{A^-} and $(f_{A^-})_1$ represent the activity coefficients of glycine anion (titration curve and reaction mixture, respectively)

For pH values < 9 , the value of K is small in comparison with those of f_{A^-} and $(f_{A^-})_1$, and can therefore be neglected. Equation (15) can then be written

$$(\text{NH}_2\text{CH}_2\text{COO}^-) = \frac{(f_{A^-})_1}{f_{A^-}} (\text{NH}_2\text{CH}_2\text{COO}^-)_1 \quad (15a)$$

When $\text{pH} > 11$, the activity coefficients, f_{A^-} and $(f_{A^-})_1$, can be neglected since their values are small in comparison with that of K . Then

$$(\text{NH}_2\text{CH}_2\text{COO}^-) = (\text{NH}_2\text{CH}_2\text{COO}^-)_1 \quad (15b)$$

On the basis of equations (15a) and (15b), the factor, F , which characterizes the primary salt effect, must be multiplied by the ratio $\frac{(f_{A^-})_1}{f_{A^-}}$ (when the pH is lower than 9) in order to obtain the factor $(F)_T$ which takes into account both the primary and the secondary salt effect

$$(F)_T = \frac{(f_{A^-})_1 f_{B^-}}{f_{X^-}}$$

Above pH 11 the factor F accounts for the primary salt effect only, being given by

$$F = \frac{f_A f_B}{f_{X^-}}$$

Since all of these activity coefficients are not known, the question of the salt effect cannot be solved quantitatively and, for the same reason, the velocity constants, independent of the salt concentration, cannot

TABLE V

pH	$k_1 \times 10^3$	Concentrations of buffer components				
		Glycine	H ₂ BO ₃	K ₂ HPO ₄	NaOH	KCl
		<i>mols</i>	<i>mols</i>	<i>mols</i>	<i>mols</i>	<i>mols</i>
10.29	7.3	↑	0.247		0.395	0.1
	8.2		0.247		0.395	0.37
10.52	8.0		0.247		0.413	0.1
	9.5			0.50	0.290	
10.89	8.8		0.247		0.437	0.1
	12.0			0.50	0.370	
11.41	9.5		0.247		0.460	0.1
	10.8			0.25	0.370	
12.10	10.0		0.247		0.480	0.1
	7.4				0.230	
12.90	10.7		0.247		0.616	0.1
	7.7				0.350	

be given. From a qualitative standpoint, however, Bronsted's postulates are satisfied. The velocity constants k_1 (see Table IV, Column 5) increase with the ionic strength, thus showing that there is positive salt catalysis. The increase in the velocity constants over the pH range of 9–11 is relatively smaller than outside of this pH range. This is due probably to the continuous disappearance of the secondary salt effect. At lower pH values, this effect resulted in an increase in the value for F .

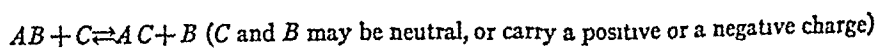
Further evidence for the existence of the positive catalytic salt effect can be demonstrated when the values for k'_1 are compared in solutions of the same pH but of different ionic strengths. The data which are given in Table V show that the increase in the concentration of KCl increases the value for k'_1 . A considerable increase in the velocity constants was found when phosphate buffers were used. This is due to the high valency of HPO_4^- and PO_4^{3-} . When, on the other hand, only glycine was used as a buffer, the velocity constants were lower than in the glycine-borate buffer mixture of higher ionic strength. (See data which are given in the last four horizontal lines of Table V.)

In concluding this discussion on the salt effect, it should be mentioned that the ionic strength does not remain constant during the reaction, since divalent and, to some extent, trivalent substituted glycine anions are produced under the described experimental conditions. It can be expected, therefore, that the velocity constants will increase during the course of the reaction. On the other hand, the decrease in pH during the reaction tends to diminish the velocity constants. It appears, therefore, that these effects compensate each other, and the constants remain only apparently unchanged.

Some increase in the velocity constants can be observed at pH values which are higher and at which the decrease of pH during the reaction does not influence the concentration of glycine anion.

The experimental evidence that only the anionic form of glycine is able to react with iodoacetate finds also some theoretical support.

Olson (17) discussed recently the mechanism of certain substitution reactions of the type



in which an addition compound, ABC , represents the first step. He showed that the existence of this intermediate complex is in accordance with the quantum mechanical conception of the chemical bond. In the case of the halogen acids, where the halogen is placed in the apex of the carbon tetrahedron, the carbon halogen bond extends beyond the carbon atom on the side away from the halogen. Therefore, the carbon face opposite to the halogen is able to react with a particle carrying unbonded electron pairs, whereas the halogen ion is ejected from the molecule.

On the basis of these considerations, an explanation offers itself that iodoacetate can react with the amino group if the nitrogen possesses an unbonded electron pair. Since in the zwitter ionic form this electron pair is shared with hydrogen ion forming —NH_3^+ , only the anionic form of glycine, where this pair is free, is able to react. The intermediate addition compound corresponds then to Brönsted's critical complex.

The author is cordially indebted to Professor Carl L. A. Schmidt for the suggestion of this problem and for his helpful interest and criticism during the work.

SUMMARY

1 The kinetics of the reaction which takes place between glycine and iodoacetic acid was studied by means of the polarographic method.

2 On the basis of kinetic equations, evidence was obtained that (a) The reaction proceeds in two steps in which the hydrogens of the amino group are consecutively replaced by the acetyl radicals, the velocity constants being in the ratio 2 : 1. (b) Only the anionic form of glycine is able to react since the velocity constants at any pH are proportional to the concentration of glycine anion. (c) The reaction is of the ionic type, showing a positive salt catalysis, which, according to Brönsted's hypothesis, involves the primary and the secondary salt effects.

3 The fact that only the glycine anion is able to react was explained as being due to the existence of an unbonded pair of electrons on the nitrogen in the NH_2 group. The NH_3^+ group, however, in which these electrons are shared by H^+ , must, therefore, be inactive.

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THE EFFECTS OF CURRENT FLOW ON BIOELECTRIC POTENTIAL

II HALICYSTIS

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A description will be given, largely in the form of string galvanometer records, of the effects of the flow of direct current, of controlled density and direction, upon the potential difference exhibited across the protoplasm of impaled cells of *Halicystis*. Some of these effects duplicate closely those of other treatments (ammonia,¹ vacuolar perfusion, and unbalanced NaCl²) and comparisons will be made with these as a basis for the explanation of the effects, not only in *Halicystis* itself, but in *Valonia*⁴ (already described) and in *Vitella*, which follows in this series.

Method

The measurements were made on cells impaled by the methods previously employed.¹ Fine glass capillaries inserted into the vacuoles served with proper external salt bridges and calomel electrodes to tap the potential difference across the protoplasm and to carry currents across it. The characteristic positive P.D. of the species (about 68 mv. with *H. Osterhoutii* and 80 mv. with *H. ovalis*) was reached in a few minutes to half an hour after impalement, but the measurements were usually made after at least a day to allow good healing of the wound around the capillary. Good results were obtained for a week or 10 days after impalement. The two species were in essential agreement as regards current flow despite their difference of sap content.⁵

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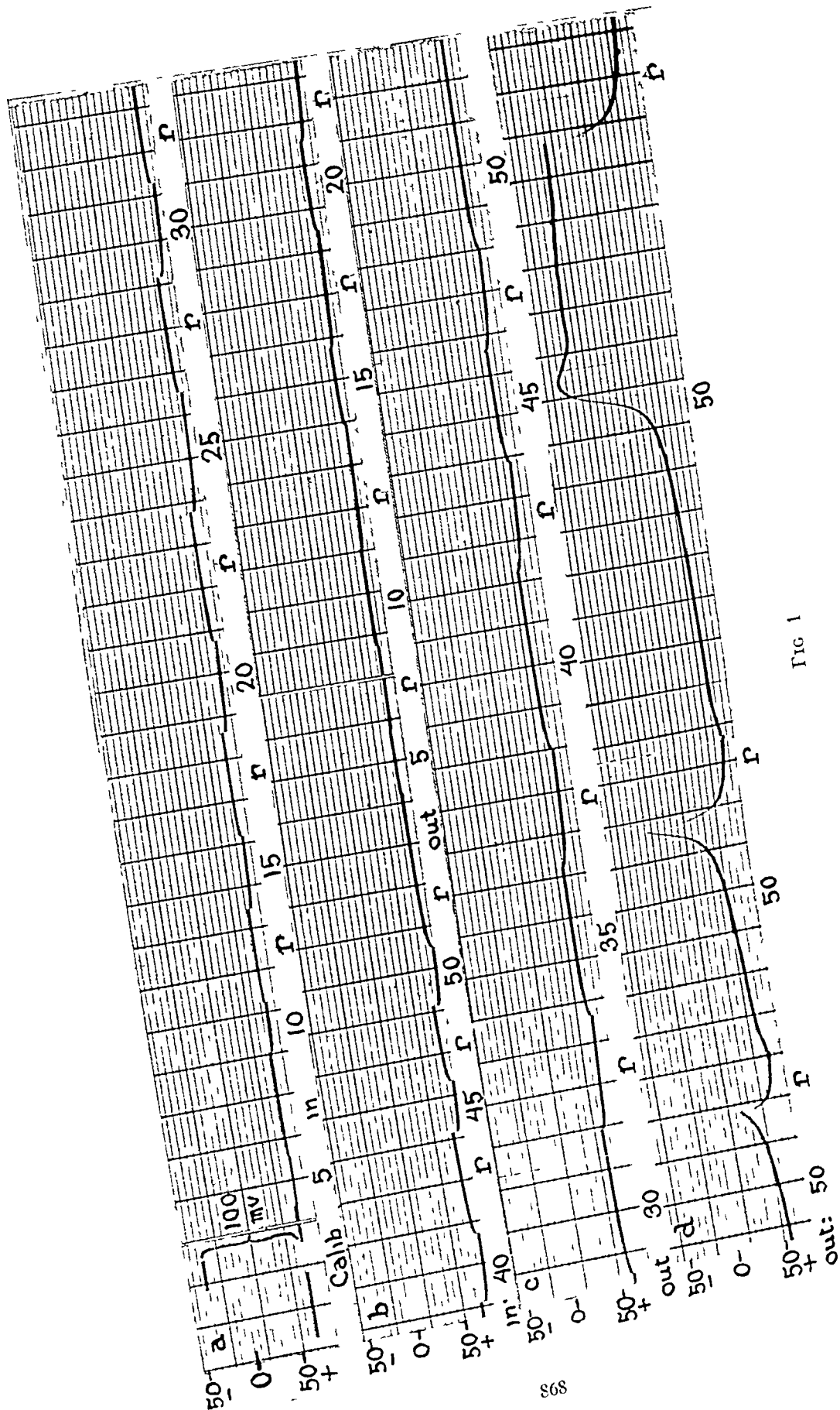


FIG 1

The potential changes are presented in the form of string galvanometer records taken by the methods described for *Valonia*⁴. These show the bioelectric potential in proper magnitude and sign at all times before, during, and after current flow. The advantages of such continuous recording outweigh the possible error introduced by the completed bridge circuit necessary to balance out the ohmic resistance of the capillary, etc. Through this circuit of course the normal bioelectric potential can discharge, producing a residual current outward across the protoplasm at all times. However, owing to the high resistance employed (twice that of the capillary) this current is so small as to be almost without effect, as will be seen in the description of experiments below. It can, if desired, be abolished by compensating the P.D. with an equal and opposite potential in the circuit; when this is done, the curves of potential change during current flow entirely resemble in time shape and magnitude those taken uncompensated, showing the residual current drain to be of negligible effect.

The values given with each record are of current density (microamperes per square centimeter of cell surface) calculated from the current sent through the bridge and the surface of the cell. The latter is estimated from caliper measurements of the cell after impalement (it shrinks noticeably on impalement by loss of sap into the capillary) and is subject to some error due to irregular cell shape. The order of magnitude is essentially correct, however. Since the threshold for reversal and other effects differs somewhat from cell to cell, the absolute value is of less importance than the current increments for a given cell. These are correct to the readings of the voltmeter connected across the bridge, i.e., to about 2 per cent. This meter checked the equality of the dry cell units comprising the input battery to the bridge, and dry cells were discarded when appreciably low in voltage.

The experimental current densities are to be regarded as in *Valonia*⁴ as changes superimposed upon the residual current, and furthermore, as the values at the beginning of current flow, before a counter E.M.F. has developed to decrease the current.

FIG. 1. Effect of direct current flow on the bioelectric potential of *Halicystis Osterhoutii* (freshly impaled). First inward currents (from sea water to sap) are passed, in increments of $5\mu\text{a}/\text{cm}^2$ up to $50\mu\text{a}$, then outward currents in the same progression. Only at $50\mu\text{a}$ is the threshold for reversal reached; reversal occurring in a sigmoid curve. Positivity is regained on interruption of outward current in a regular curve without inflection from various points on the reversal process (Record d).

Sensitivity about 7 mv per horizontal division; 100 mv calibration on Record a and 50 mv + and - indicated on each record. Time marks 1 second apart. When the current is shut off, there is a residual current (r) produced by the cell, which in this case amounts to about $0.33\mu\text{a}/\text{cm}^2$ (outward). In indicates passage of positive current inward (from sea water to sap); out means outward current with densities as indicated in μa per cm^2 of cell surface.

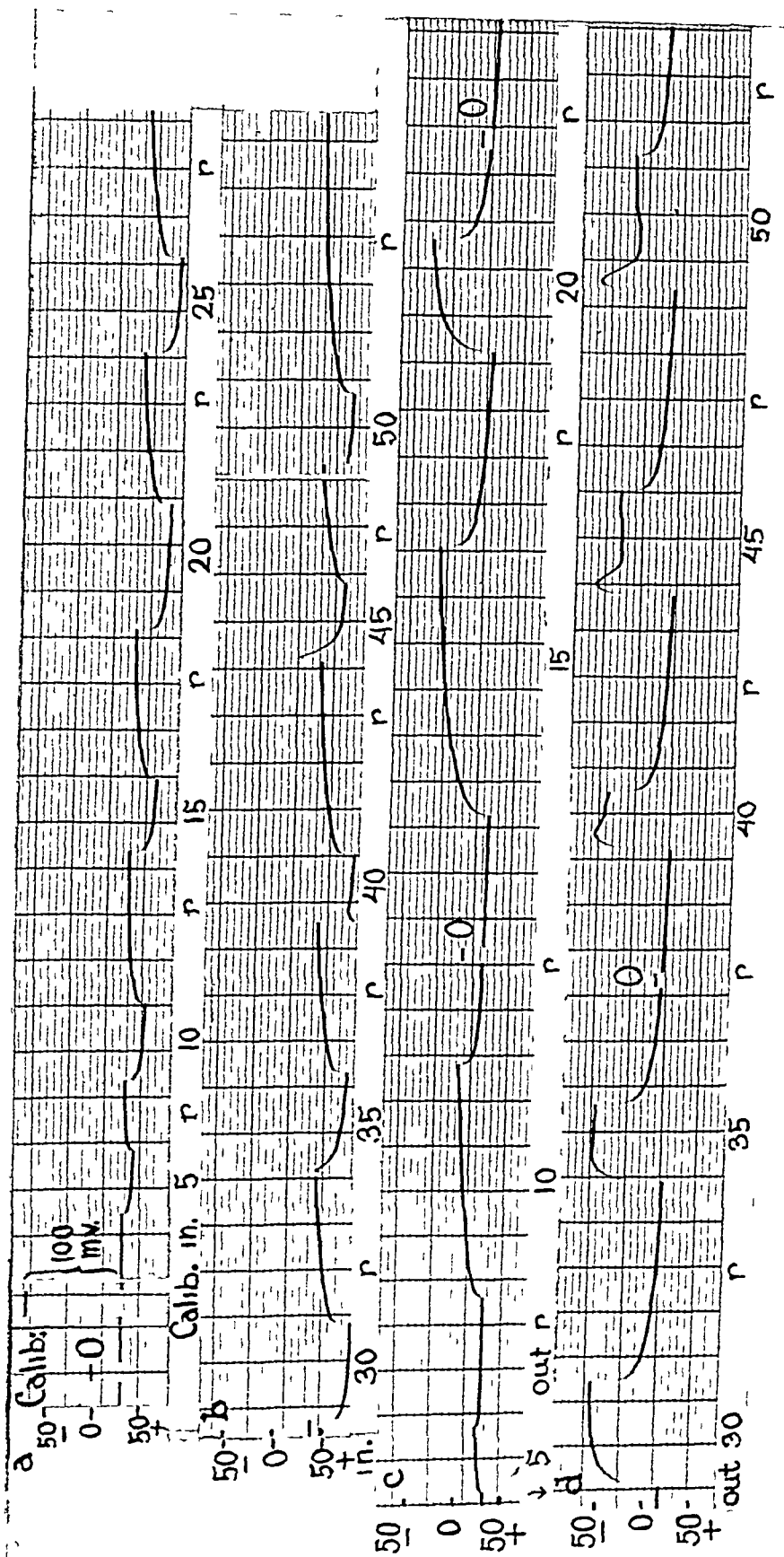


FIG 2

Effects of Current Flow

It would perhaps be more logical to begin with the effects of currents passed outward across the protoplasm since this is the direction in which the normal bioelectric potential of *Halicysts* tends to discharge when short circuited. But these become rather complicated at the higher current densities. The effects of currents in the opposite direction will therefore be first considered because they involve only the simpler phenomena which may be designated for convenience as polarizability (without immediate prejudice as to their real nature i.e. whether a static or polarization capacity is displayed).

Inward Currents—When potentials opposite to and greater than the normal bioelectric potential are applied to the cell positive currents pass inward across the protoplasm from sea water to sap. Over the range of current density studied (from 1 to 100 $\mu\text{A}/\text{cm}^2$ of cell surface), the effects of such currents are comparatively simple. They increase the positive potential to a small extent and almost in proportion to the current density up to a maximum increase of 20 to 30 mv. This increase occurs in a smooth regular curve often occupying well over a second for approximate completion and the positive potential remains augmented as long as the current continues to flow. When it is stopped the original value is regained, again in a slow smooth curve. Typical responses are shown in Fig. 1 for a characteristic range of current densities.

There appears to be an upper level beyond which the positive potential cannot be increased at about 100 mv. maximum r.p. The significance of this is not fully clear, but it is remarkably similar (at least in *H. Osterhouti*) to an effect of more alkaline sea water, which will be

FIG. 2. Effect of acidified sea water (pH 5.0) in increasing the effects of current flow in *Halicysts Osterhouti*. The bioelectric potential is decreased (temporarily) to about 30 mv. positive upon this as a base the polarizations are now much increased in magnitude the r.p. being increased to 40 mv. or more by inward currents. On the other hand outward current now produces reversed potential, but without a marked threshold the curves being regular and without inflection, until the 'cusp' begins to appear at about 40 μA . Irregularities also appear with inward currents above 30 $\mu\text{A}/\text{cm}^2$.

Sensitivity about 1.5 mv. per horizontal division calibration on record a and 50 mv. + and - shown on each record. Time marks 1 second apart. Residual current (r) about 0.2 $\mu\text{A}/\text{cm}^2$. Designations as in Fig. 1.

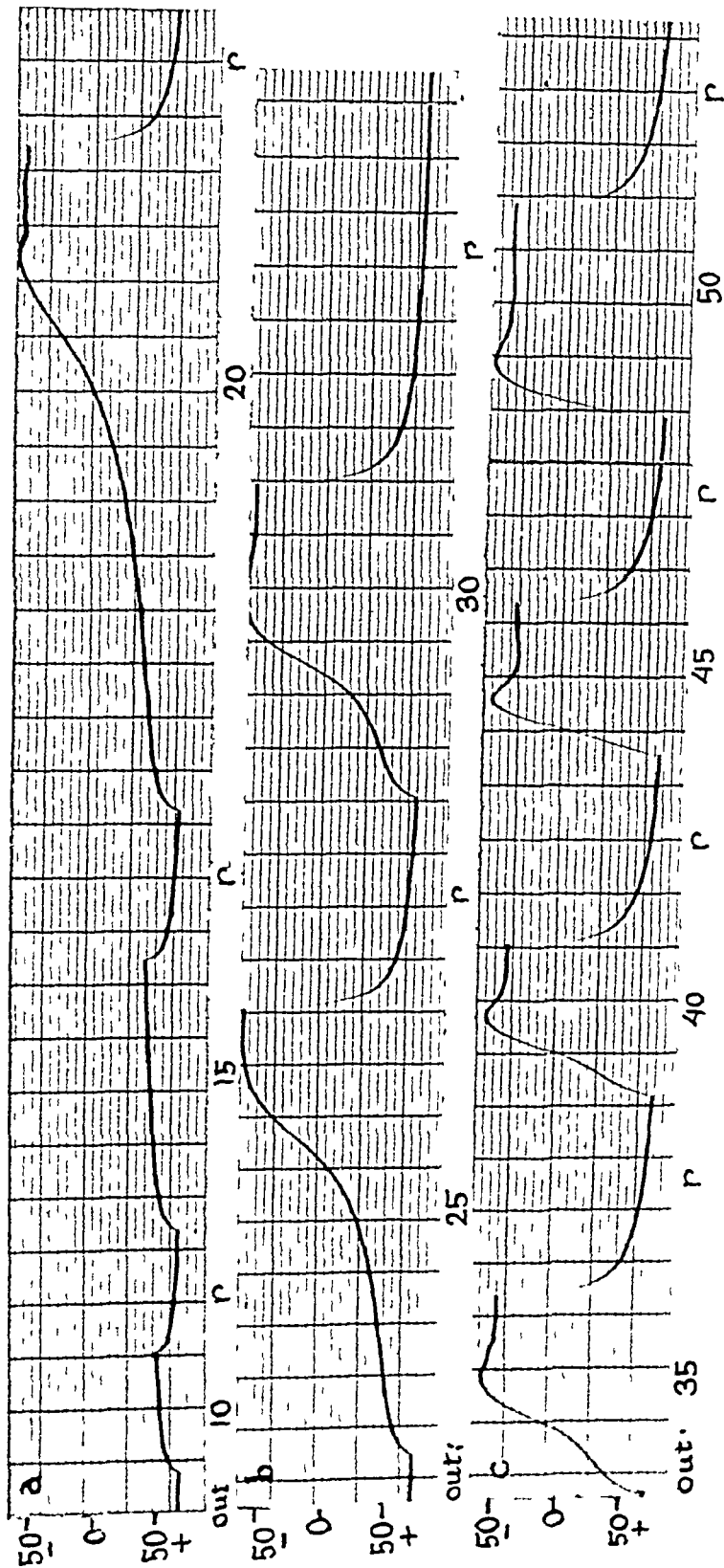


FIG 3

mentioned later in the Discussion. Possibly connected with this pH effect is also the much greater response to inward current which occurs when this species is exposed to sea water acidified to pH 5.0. The $P.D.$ is decreased to some 30 or 40 mv. by this treatment. The polarizability is also greatly increased to inward currents, as shown in Fig. 2. It appears that the $P.D.$, starting at a lower level, is now more readily increased, and the polarization curves are of greater magnitude. In other words, the effective resistance (which is determined by the amount of back $E.M.F.$) is greater. A similar increase of polarizability by lowered pH has been found in *Idioneis*. The $P.D.$ of *Idioneis*, on the other hand, is not appreciably reduced, nor is its polarizability greatly affected, by the pH of the sea water. (See note, p. 896.)

While it is not the purpose of this paper to discuss the effective capacity of the protoplasm, it is obvious from the extended times of charge and discharge that it is very large.

Aside from these regular 'polarizations,' which are the chief effect of inward currents, there are two other phenomena produced by inward currents. One, the recovery of positivity after reversal of $P.D.$ will be discussed after the effects of outward currents and of ammonia, described below. The other, an occasional production of rhythmically recurring potential changes, is reserved for a later paper.

Outward Currents—As pointed out above, the normal bioelectric potential tends to discharge in a positive current outward across the protoplasm, from sap to sea water, when connected into a completed circuit. The resistance in series with the cell in the bridge was usually quite large, generally over 100,000 ohms, so that this 'residual current' was not over 0.7 to 0.8 μa giving a density of about 0.4 $\mu a/cm^2$.

FIG. 3. The effect of increasing outward current density upon the speed of reversal of $P.D.$ in *Halcyotis Osterhoutii*. This is the same cell as used in Fig. 1 but after recovery from impalement so that the threshold for reversal is lowered to 20 $\mu a/cm$. (15 μa was sufficient to reverse with very long flow.) As the current is increased by 5 μa increments the speed increases (although the total negative $P.D.$ is not increased indeed is somewhat lower at high densities). The sigmoid character is less and less pronounced and is almost lost at 50 μa . The recovery curve in every case is regular and without inflection.

Sensitivity 6.6 mv. per horizontal division. 50 mv. + and - being indicated on each record. Time marks 1 second apart. Residual current (r) about 0.33 $\mu a/cm$. Designations as in Fig. 1.

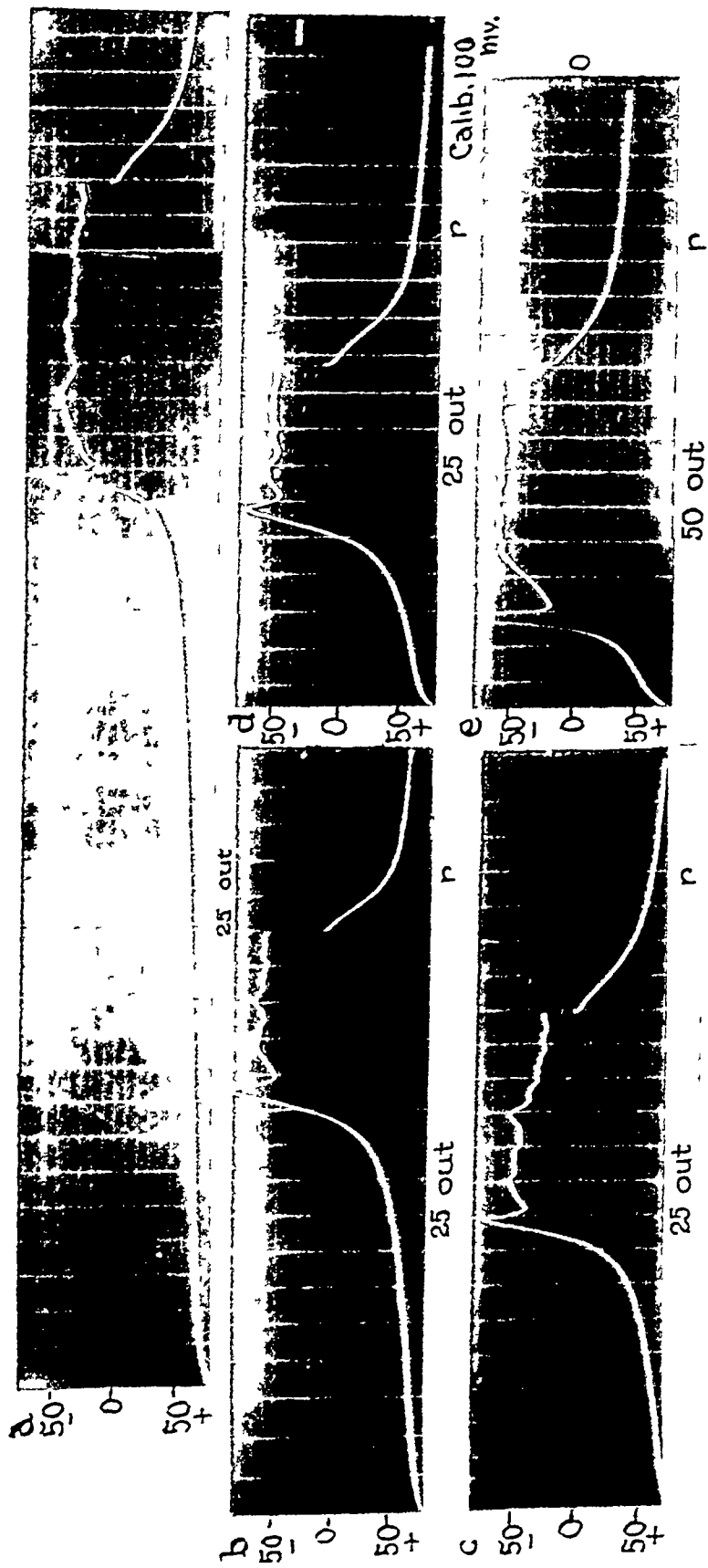


Fig. 4

through the average cell (diameter 0.8 cm, surface about 2 cm²). It was repeatedly found that the cell may be left connected into the bridge for several days with this current flowing, without appreciably altering the P.D. Indeed, much larger currents (up to ten times this value) may be drawn in this manner, through capillaries of lower resistance (10,000 ohms) and negligible external resistances (galvanometer), with only a slight effect on P.D. (1 or 2 mv reduction).

This may best be seen under controlled conditions in Fig. 1 which shows the effects of increased outward current in the bridge, beginning with the residual current and increasing by $5 \mu\text{A}$ steps up to $50 \mu\text{A}/\text{cm}^2$. The response at low current densities greatly resembles that produced by inward currents, except that the normal P.D. is now slightly decreased, instead of increased. The decrease again takes place in a regular curve, which reaches a steady value: the latter roughly proportional to the current density. This may be called normal polarization; the total change of potential is again about 10 to 20 mv, reducing the P.D. to some 50 or 60 mv positive. The latter value, however, is no longer a limiting potential beyond which no further change occurs; it is rather a threshold for much larger effects, of a quite different sort from normal polarization.

The new type of response is shown in Fig. 1 d (continuing from Fig. 1 c). This increment of current begins much as the previous one, but instead of flattening out at a steady value, the P.D. curve begins to inflect upward, approaches zero more and more rapidly, and finally passes it so as to reverse the sign and become 40 or 50 mv negative. It is obvious that this particular increment of current, although of the same size as previous ones, produces an effect (100 mv change) out

FIG. 4. Records showing the characteristic form of the reversal curve in *Halobacteria* *Osterhoutii* on succeeding current flows of threshold density (outward current). From a through d the same density ($25 \mu\text{A}/\text{cm}^2$) was passed, reversal becoming more rapid on successive flows. In e this density was doubled to $50 \mu\text{A}/\text{cm}^2$, greatly increasing the speed of rise and the sharpness of the cusp, but not greatly increasing the negative P.D. attained. An inflection in the recovery curve characterizes all these records, with an incomplete recovery after the highest current (in e) probably due to injury. Sensitivities as shown with calibration on d. Time marks 1 second apart. Residual current (r) about $0.5 \mu\text{A}/\text{cm}^2$ (outward). Designations as in Fig. 1.

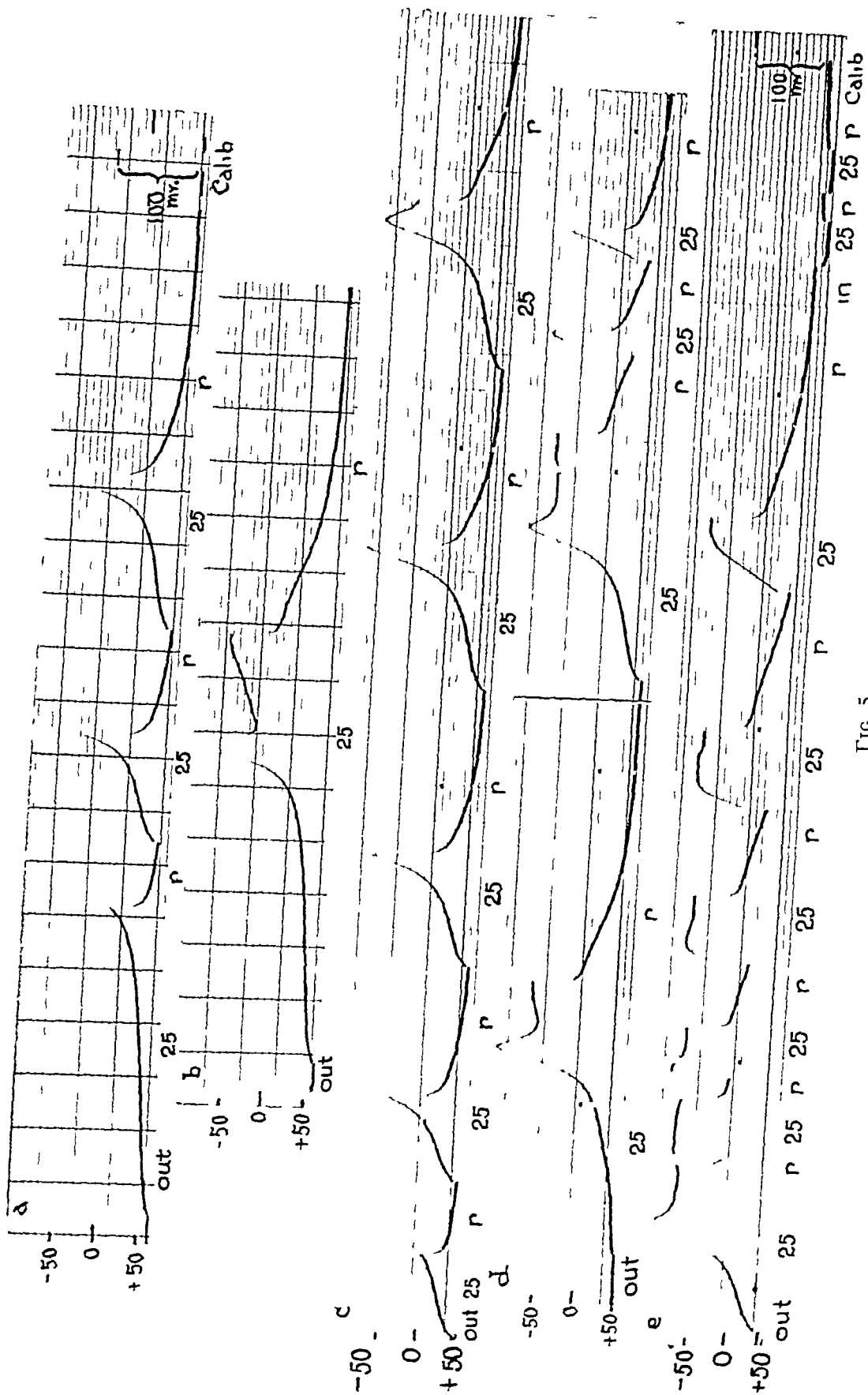


FIG 5

of all proportion to its size, and greater than all the previous total current flow

If the current is now stopped, recovery of positive P/D occurs, in a regular curve, often without an inflection (Fig. 1) although this sometimes occurs (Fig. 4)

Fig. 3 shows other reversals at increasing current densities. It is seen as would be expected, that reversal is more rapid the higher the current density, eventually becoming fast enough to smooth out most of the inflection and to destroy the typical S shape of the reversal at threshold densities. It should also be noted that only slight increases of negativity are produced by further increases of current above this threshold, once reversal has occurred: equal increments produce only relatively small changes of P/D . One feature, moreover, is almost invariably found: the cusp occurring just after reversal, due to a temporary recession of negativity, followed by an increase again. The irregularly wavering negative value that follows is also characteristic. Examples are shown in Fig. 4.

Fig. 5 shows the effects of interrupting the flow of current at densities well above the reversal threshold. The P/D immediately starts to recover as soon as the current stops, no matter where along the reversal curve this occurs. It is evident therefore that the current flow does not here initiate a process, which at some critical point goes on to completion independently: but its flow is necessary to maintain the altered P/D at all times. In this it differs strikingly from the situation

FIG. 5. The effects of current flow on the bioelectric potential of *Halocystis Osterhoutii* showing time relations by interrupting the flow of outward current at various points during its course: before and after reversal has been produced. Records *a* and *b* employed a current density of $25\mu\text{a}/\text{cm}^2$ passing outward across the protoplasm. The residual (outward) current was about $0.17\mu\text{a}/\text{cm}^2$ of cell surface or less than 1 per cent of this experimental density. The sensitivity in *a* and *b* is about 8.5 mv. per horizontal division. 50 mv. marks and 100 mv. calibration are shown. Records *c*, *d* and *e* were taken with another cell and show the same effects with interruptions continued still longer after reversal. The outward current density here is $25\mu\text{a}/\text{cm}^2$ of cell surface throughout: the residual (outward) current (r) is $0.25\mu\text{a}/\text{cm}^2$ (or 1 per cent of the experimental current). Sensitivity on *c*, *d* and *e* about 10 mv. per division as shown by calibration and 50 mv. marks. Time marks 1 second apart. Designations as in Fig. 1. An automatic zero device interrupts the curves on Records *c*, *d* and *e* every 9 seconds.

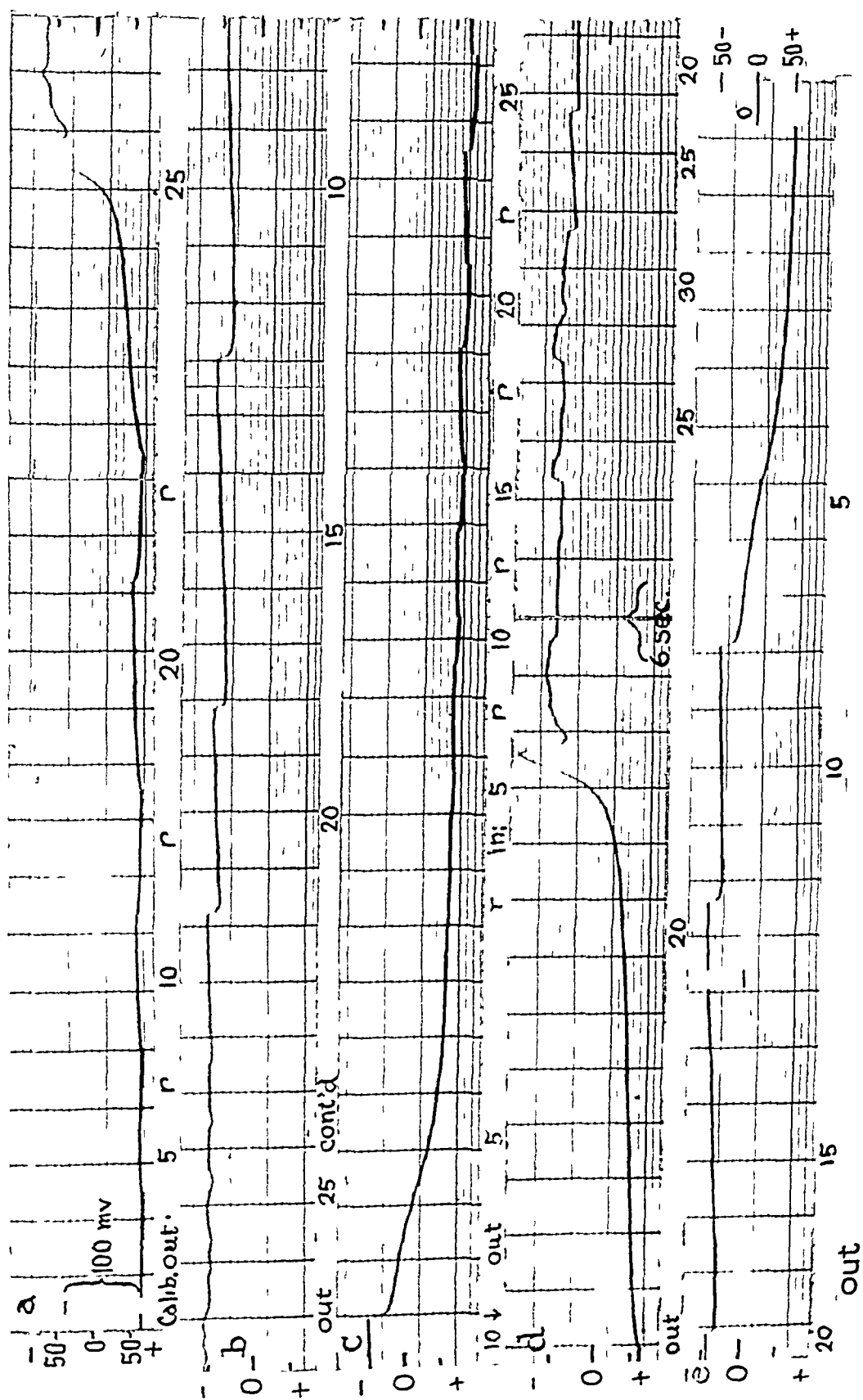


Fig 6

in *Nitella*, where the change in $P D$ (action current) goes on through its complete cycle, if carried to a certain point, but it will be shown below that conditions in *Halicystis* itself may likewise be so changed that the latter type of response may occur. Indeed, even under normal conditions, although current flow is necessary to maintain as well as produce the reversed $P D$, it is found that the same current density is not necessary to maintain it at all stages of the curve. This is shown by a different type of experiment, in which the current is decreased in equal steps without interrupting it to allow depolarization or recovery to occur between each density value.

Fig. 6, dealing with such an experiment, shows first of all the effect of increasing the current. The small effects of the first increments, the large change at the threshold value and the relatively small changes again thereafter (but with cusps) are to be clearly seen.

The current is now decreased in equal decrements. The negativity decreases for each decrement, but the $P D$ still remains negative at a considerably lower current density than was originally necessary to produce reversal. This is a definite hysteresis since only at below half the original threshold density does recovery occur, and here at a much slower rate than in the absence of current flow. That the $P D$ actually recovers during continued current flow is the best argument for the reality of the hysteretic effect, since it disposes of the possibility that small currents, if allowed to flow long enough, would produce reversal. For even after reversal has been produced by a larger current, recovery occurs while a small current flows outward.

FIG. 6. The effect of decreasing the current density during continued flow of outward current which has reversed the $P D$ of *Halicystis Osterhoutii*. The threshold is first at $25\mu\text{a}/\text{cm}^2$ later at $20\mu\text{a}$ (long flows of $15\mu\text{a}$ produced no reversal). 15 and even $10\mu\text{a}$ were however quite sufficient to maintain negativity once this had been produced by larger currents; only on a decrease of density to $5\mu\text{a}$ did the $P D$ become again positive here with a pronounced lag and inflection. In d the current was increased from the threshold density of $20\mu\text{a}$ to 25 and 30 , initial cusps were the only effect the $P D$ being scarcely altered permanently. Successive decreases again maintained negativity down to $10\mu\text{a}$ with slow recovery of positivity during the flow of $5\mu\text{a}$ (a break of 6 seconds appears in Record d as indicated).

Sensitivity about 10 mv per horizontal division; calibration on a 50 mv ; + and — marks on each record. Time marks 1 second apart; residual current (r) about $0.4\mu\text{a}/\text{cm}^2$. Designations as in Fig. 1.

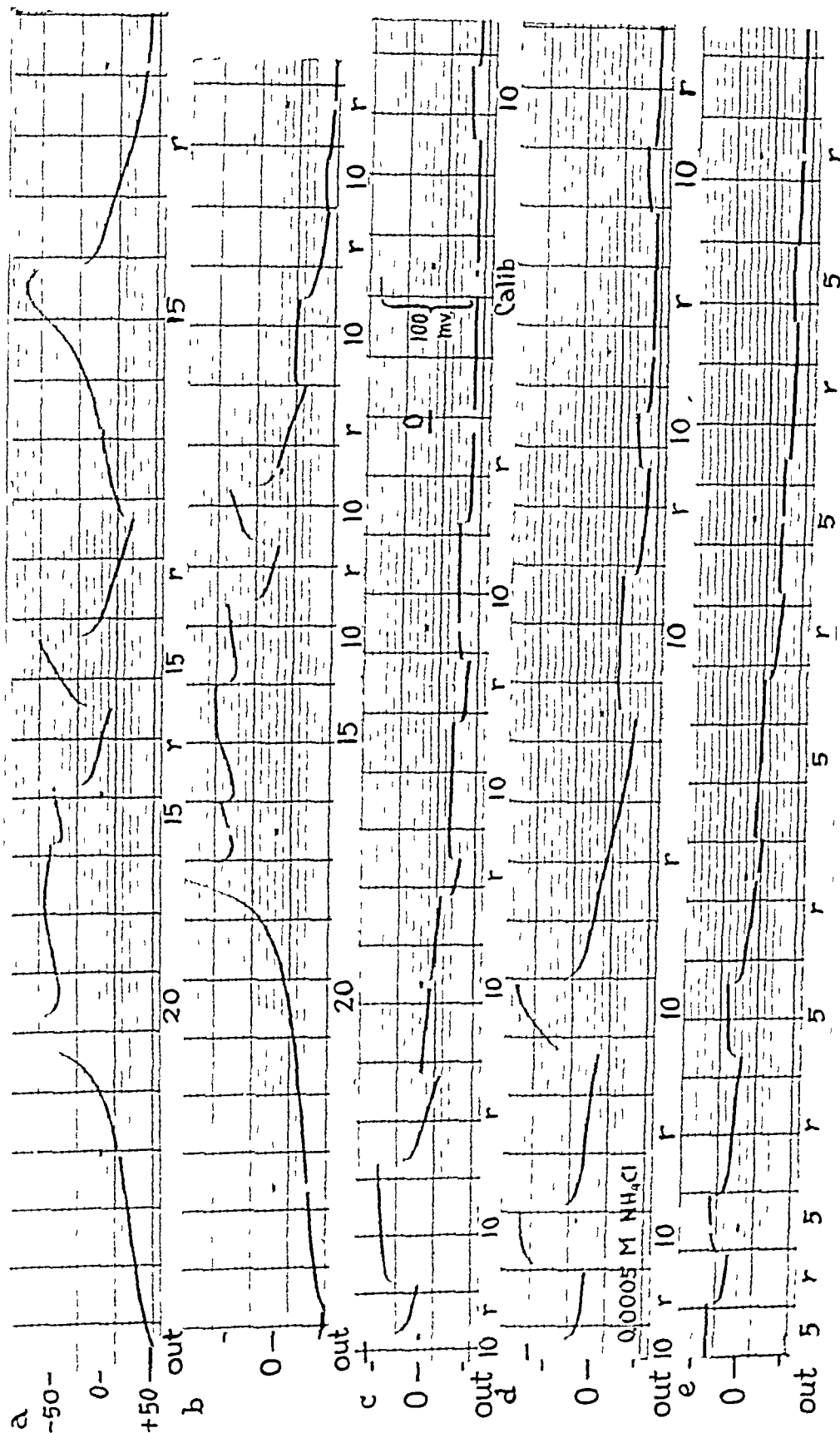


FIG 7

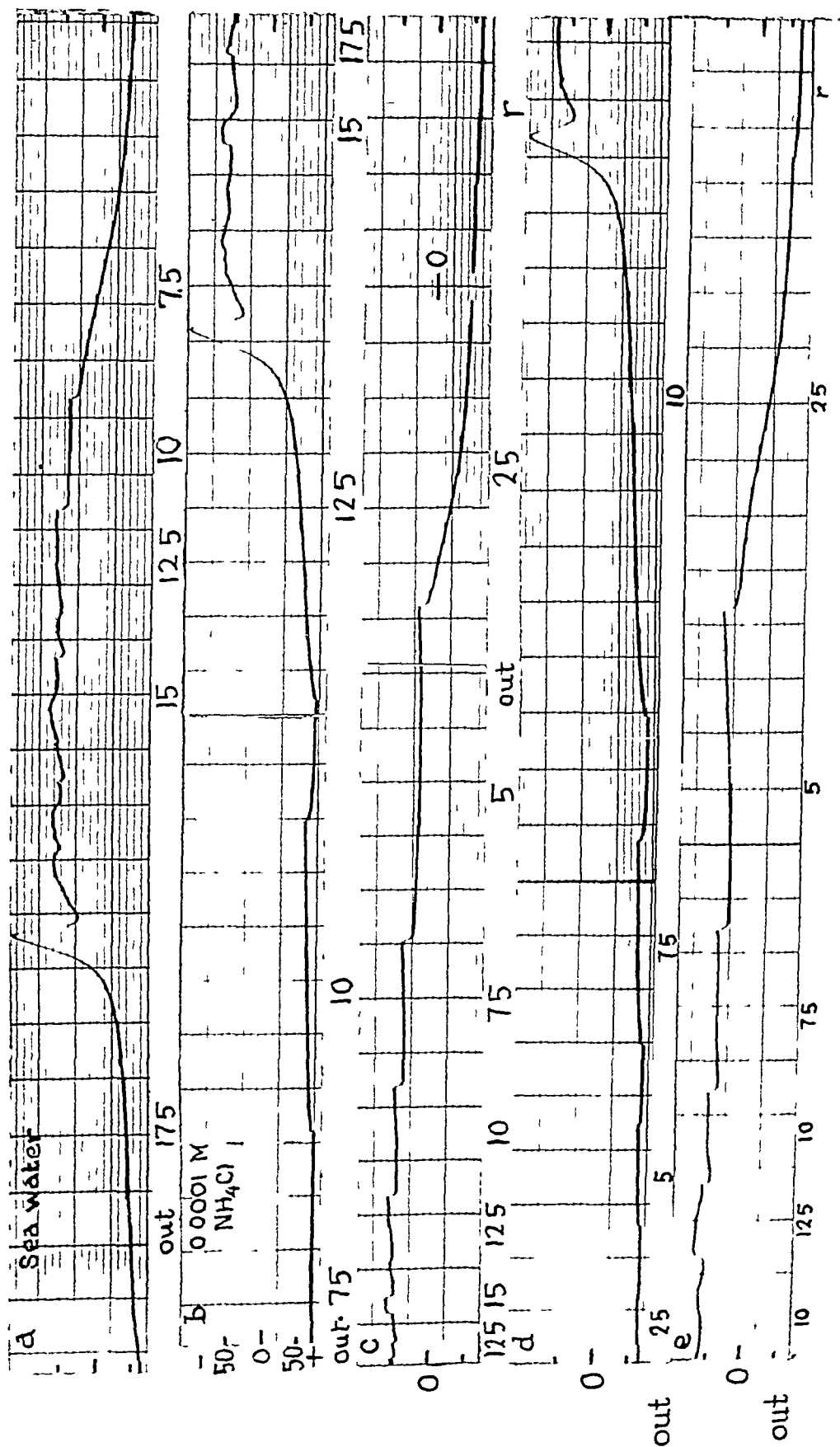


FIG 8

the effects of ammonia.¹ The common characteristics may now be indicated. These are

1 Small effect of sub threshold treatments (small current density or low ammonia concentration)

2 A threshold at which a very large change occurs, and the P/D reverses in sign. The S shaped curve is found in both treatments

3 A pronounced cusp following closely after reversal, and an irregularly wavering negative value thereafter

4 Smaller cusps, and relatively small changes of negative P/D with current densities or ammonia concentrations above the threshold value

5 Maintenance of the negative P/D by currents or ammonia concentrations smaller than the original threshold values

6 Recovery of positivity with still lower ammonia concentrations or current density

7 General similarity of the absolute P/D values, both positive and negative, produced by the two treatments and characterizing the threshold

Typical ammonia curves as previously published,¹ closely resemble the galvanometer records of outward current flow effects (e.g. Fig. 4). The chief differences lie in the greater speed of the current flow phenomena, which are finished in seconds, instead of minutes as with ammonia and the cusps, present with sub threshold ammonia concentrations but absent with sub threshold currents

FIG. 8. The effect of very low ammonia concentrations in lowering the threshold of current density necessary to reverse the P/D of *H. Osterhoutii* and to maintain its negativity. Record *a* shows a typical reversal of P/D without ammonia; negative P/D being produced first at $17.5 \mu\text{A}$ outward current per square centimeter of cell surface and being maintained by outward currents down to $7.5 \mu\text{A}/\text{cm}^2$ at which density the P/D becomes again positive even though the outward current continues to flow. The cell is then exposed to $0.0001 \text{ M NH}_4\text{Cl}$ in sea water at pH 8.0 (Record *b*). This alone scarcely affects the P/D but the latter is caused to reverse with the passage of considerably lower outward currents i.e. 12.5 and eventually $10 \mu\text{A}/\text{cm}^2$. Furthermore as the currents are decreased the P/D remains negative down to lower densities becoming positive only at $2.5 \mu\text{A}/\text{cm}^2$ outward current (here with a long delay).

Sensitivity 10 mV per horizontal division; zero and 50 mV + and - being shown on each record. Time marks 1 second apart. Residual current (r) not over $0.17 \mu\text{A}/\text{cm}^2$. Designations as in Fig. 1.

The formal resemblances are so striking as to suggest that the effects of outward current flow may be the same as those of ammonia, and may operate either in the same way, or at least upon the same structures and mechanisms of the protoplasm. The two treatments were therefore combined, to see if they were additive or mutually helpful in producing similar effects.

Combined Treatment with Ammonia and Current Flow

(A) *Ammonia and Outward Current*—The cells were first tested with current flow to determine the threshold for reversal of P D. They were then exposed to sea water containing low concentrations of ammonia (0.0001 M to 0.0005 M NH_4Cl in sea water at pH 8.1), insufficient alone to produce reversal or, indeed, any great lowering of positive P D. Current was then again passed during this ammonia exposure, and it was found that inward currents still produced an increase of P D, and outward a decrease, with eventual reversal. But the threshold of current density for reversal was now reduced to almost half the previous value found in normal sea water (Fig. 8). The greater the ammonia concentration the smaller the threshold current (Figs. 9 and 10). Finally, at a point somewhat below the threshold for ammonia alone (0.001 to 0.003 M NH_4Cl) it was found that a very small outward current (e.g. $5 \mu\text{a}/\text{cm}^2$) was now sufficient to produce reversal, which furthermore persisted when the current was stopped (Fig. 11).

Either at this point or when reversal had been produced by ammonia alone, further passage of outward current produced very little further effect, giving only small cusps and wavering negative values with each increment (Fig. 10).

All these curves resemble those without ammonia except that the threshold for reversal is greatly lowered and the P D remains reversed without continued flow of current. Thus it appears that not only do the effects of ammonia and current flow show a formal resemblance in most characters, but the two treatments assist each other in producing the same effects. They are not, however, additive in the sense that the full effects of one can be superimposed upon the other, i.e. if reversal has already been produced by ammonia, the usual large effects of outward current are absent, and are only such as would follow from

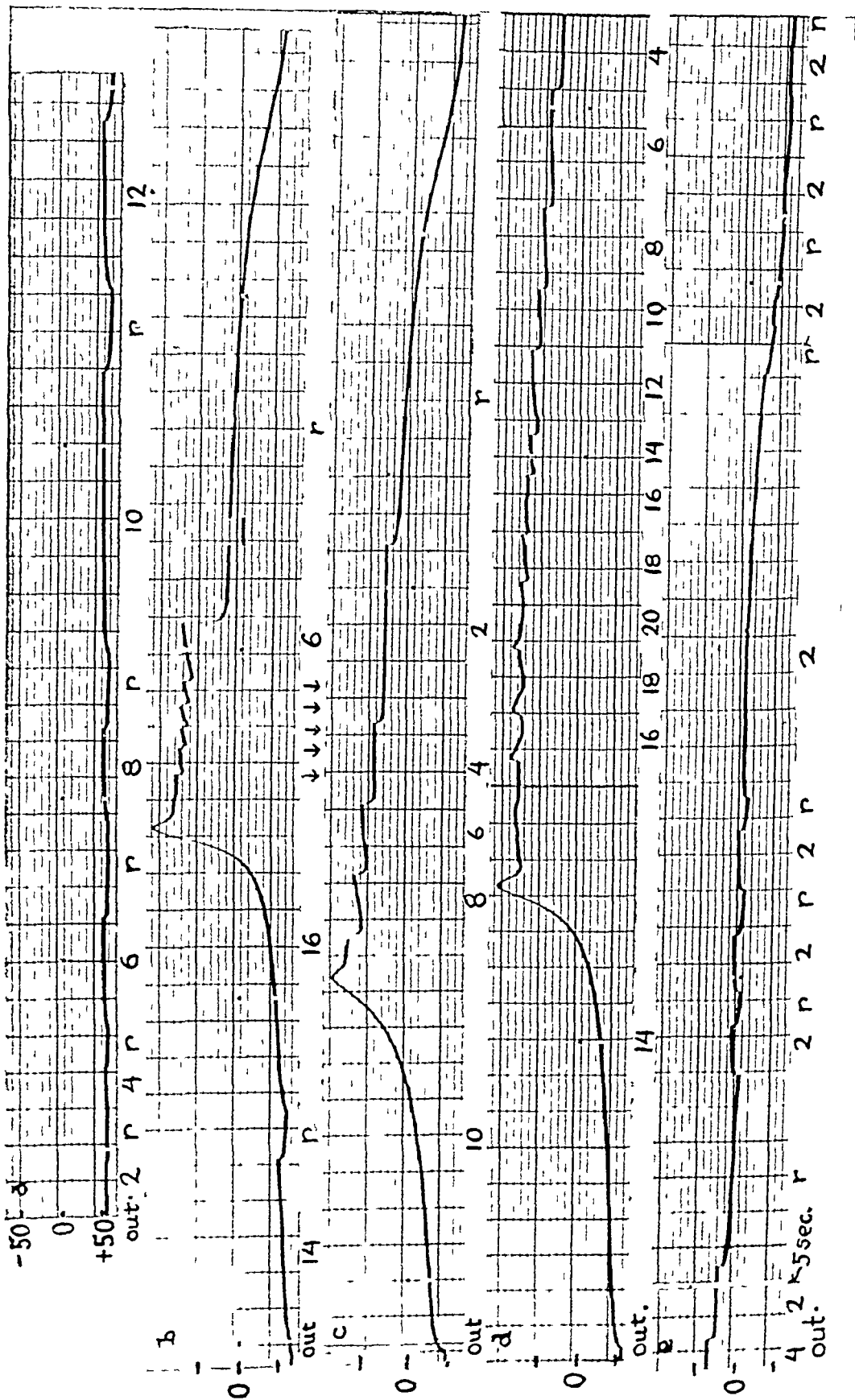


FIG 10

current increments above the threshold for reversal by current itself (cf Fig 6). They thus appear to have the same limits, and may be operating upon the same mechanism. Whether they operate in the same manner will be considered in the Discussion.

(B) *Ammonia and Inward Current*—It remains to inquire whether inward currents, which have an effect opposite to those outwardly directed within the narrow limits of simple polarization, may also counteract the large effects of ammonia. This was found to be the case.

The first experiment consisted in adding ammonia to the sea water while a large current (25 to 50 $\mu\text{A}/\text{cm}^2$) was passing inward across the protoplasm. It was found that considerable quantities of ammonia (up to 0.005 or 0.01 M NH_4Cl) could be so added to the sea water, without causing reversal although this concentration is well above the usual threshold. This was shown by the fact that when the current was stopped, reversal promptly occurred. Thus inward currents inhibit the production of ammonia effects.

They also counteract them when produced, causing recovery from negativity. This is shown in Fig 13, in which a cell, with P.D. already reversed by NH_4Cl is exposed to increasing inward currents. Small densities have little effect, moderate ones decrease the negative P.D.,

FIG 10 The effect of current flow on the potential of *Halicystis Osterhoutii* in the presence of ammonia (0.0015 M NH_4Cl in sea water at pH 8.0) just below the threshold for maintenance of negative P.D. Reversal occurs first at 16 μA outward flow and eventually at 10 μA it is maintained down to 2 μA recovery occurring extremely slowly with only the residual current (r) flowing. If recovery has not proceeded too far 2 μA outward flow tends to restore negativity but the more positive the P.D. the less effective it is recovery eventually occurring (Record e) while this small current flows. In b the current is decreased from 16 to 6 $\mu\text{A}/\text{cm}^2$ in equal decrements of 2 μA indicated by arrows. Record d shows the very slight effect of increments or decrements upon the negative P.D. when the current is above a certain value (e.g. above 12 or 14 $\mu\text{A}/\text{cm}^2$) as compared with the larger effects somewhat below this and the very large effect at the threshold for recovery. A break of 5 seconds occurs in Record c.

Sensitivity about 8 mV per horizontal division zero and 50 mV + and - being indicated on each record with frequent insertions of zero throughout. Time marks 1 second apart. Residual current (r) about 0.25 $\mu\text{A}/\text{cm}^2$ (outward) when the P.D. is fully positive correspondingly less or inward when P.D. is lower or negative. Designations as in Fig 1.

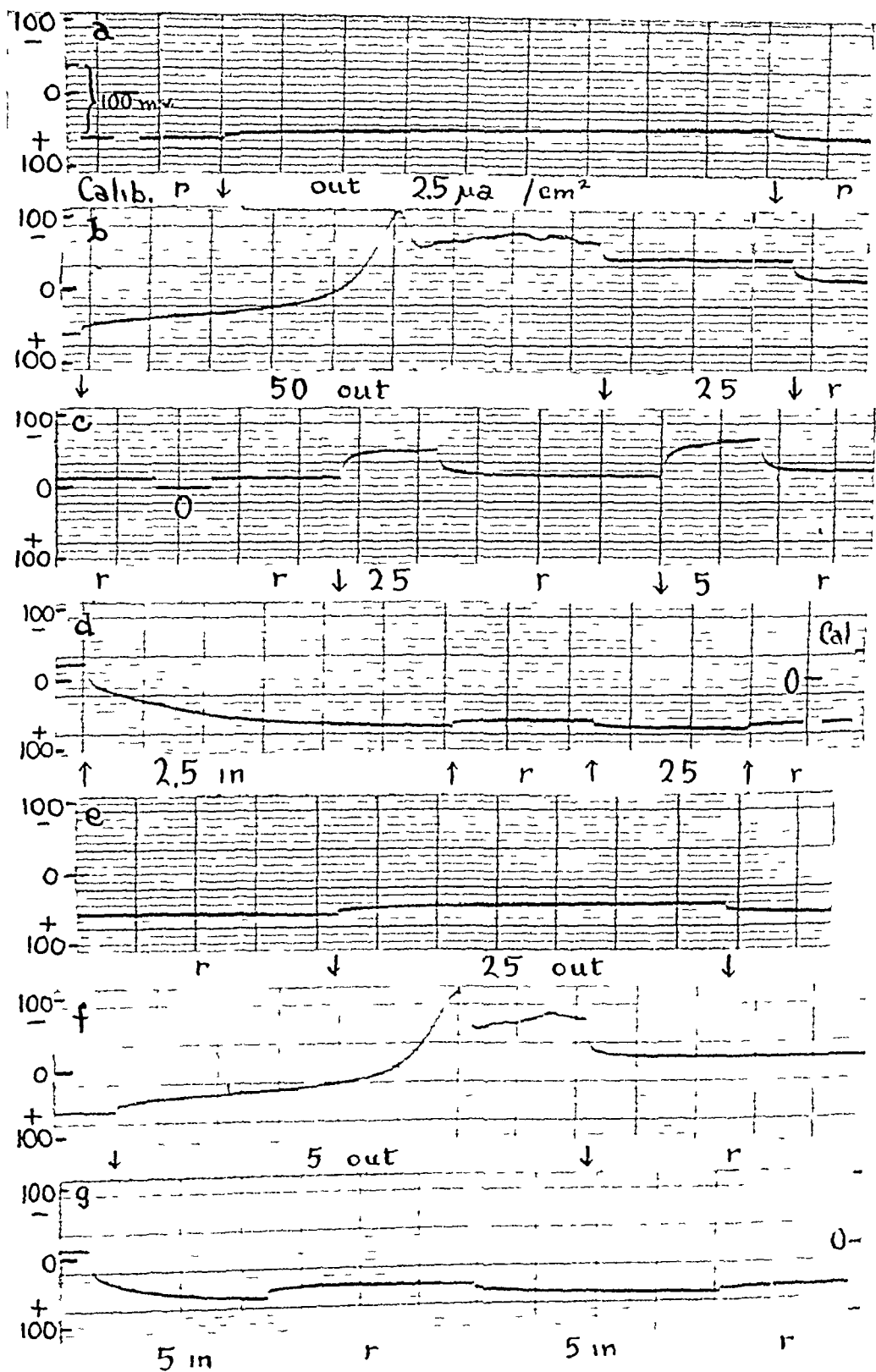


FIG 11

and large ones cause recovery of positive $P D$ for as long as they flow, when they are stopped, reversal again occurs, except at the very highest densities which finally produce positive $P D$ in Fig 13, but not in Fig 14, where a higher concentration of NH_4Cl was used. But at slightly sub threshold ammonia concentrations, hysteretic effects are again found. Fig 11 shows the complete picture, with a very small outward current causing reversal, which continues when the current is stopped, then an inward current is passed. The $P D$ is decreased by small currents and caused to recover to positive values by slightly larger ones. It then remains positive when the inward current is stopped. A very small current in either direction thus suffices to push the $P D$ to one side or the other of zero, in both cases the reaction goes on to completion and does not stay at intermediate values. This is best shown in Fig 13, where an inward current is passed for a varying period in a cell already reversed. If the current is passed only a short time, and recovery is not carried far the $P D$ returns to negative values when the current is stopped, but if it is passed for a slightly longer time and recovery goes a little farther (e.g. about to zero $P D$), it is then completed, whether the current flows or not. The ammonia concentration has to be rather carefully adjusted to give such a

FIG 11 Effect of a critical ammonia concentration on the same cell used in Figs 8 and 9. It is now exposed to 0.001 M NH_4Cl in sea water at pH 8.0. While this is not quite sufficient to produce reversal of $P D$ alone it maintains negativity after a small current flow has passed outward. $2.5\mu A$ is insufficient (Record *a*) but $5\mu A$ is adequate to do this: the $P D$ remaining reversed when the current is stopped (Records *b*, *c*). Second passages of these currents produce only the smaller polarizations of Record *c*. $2.5\mu A$ passed inward however produces reversion of positive $P D$ which persists on cessation of current and also during $2.5\mu A$ outward current (Record *e*). It is again reversed by $5\mu A$ outward current (*f*) while $5\mu A$ inward causes recovery of positivity (Record *g*) more rapidly than with $2.5\mu A$ inward. A second passage produces only slight polarization.

The $P D$ is now evidently metastable and can be maintained in either positive or negative values: a small current flow sufficing to drive it to either side of zero.

Sensitivity about 11 mV per horizontal division: zero and 100 mV + and - indicated on each record with 100 mV calibrations on *a* and *d*. Residual current (*r*) not over $0.17\mu A/cm$ outward when $P D$ is positive or about $0.05\mu A/cm^2$ inward when $P D$ is negative. Time marks 1 second apart. Designations as in Fig 1. In some cases outward current designated by a downward arrow, inward current by an upward arrow.

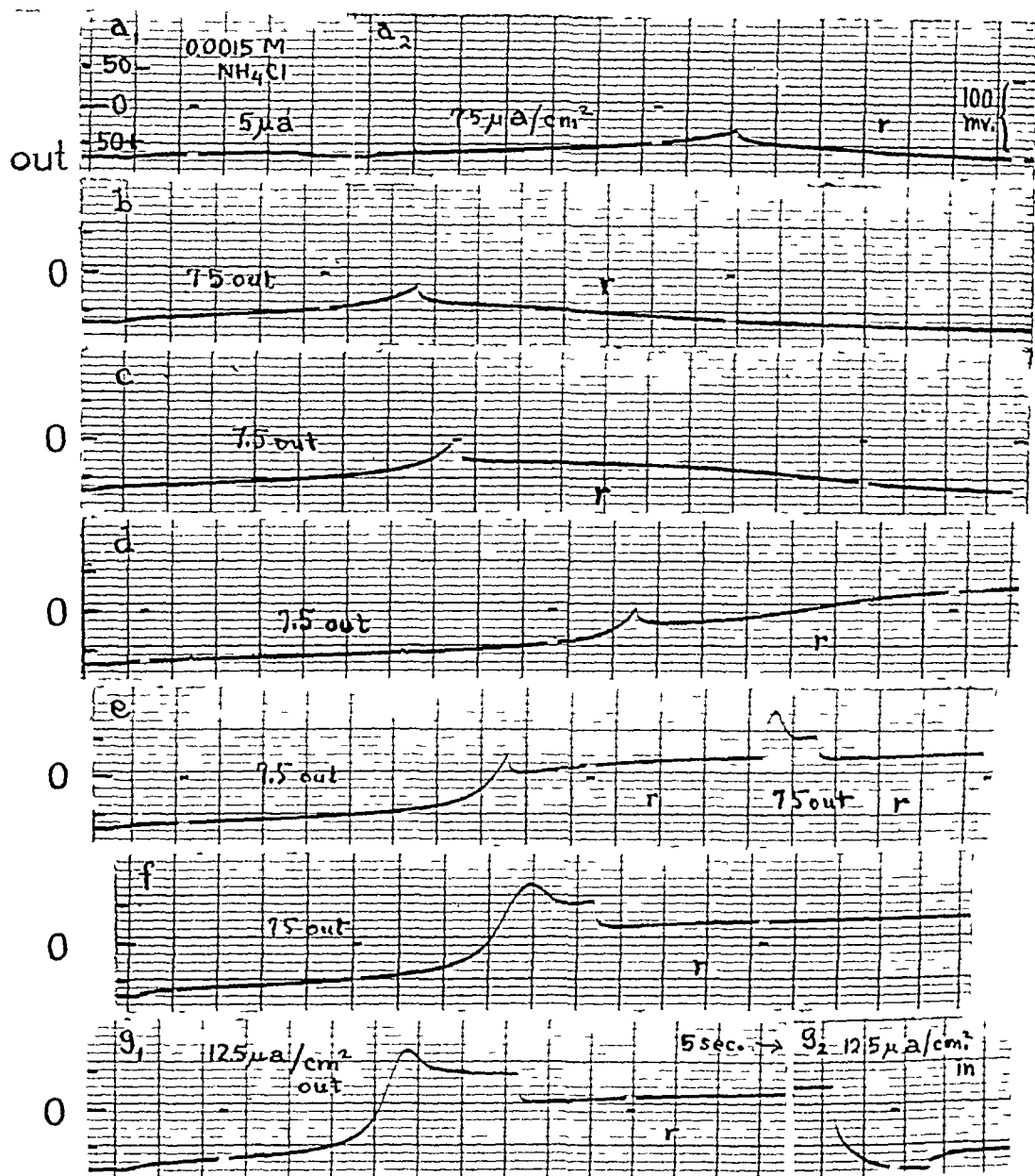


FIG 12 The effect of interrupted outward current flow on the bioelectric potential of *Halicystis Osierhoutii* in the presence of a nearly borderline ammonia concentration (0.0015 M NH_4Cl in sea water pH 8.0). This concentration, of itself not quite sufficient to reverse the P.D. of this cell, maintains a permanent negative P.D. when aided by sufficiently long flow of outward current having a critical density here of $75 \mu\text{A/cm}^2$ of cell surface. (In Record a, $5 \mu\text{A/cm}^2$ is temporarily passed, this is insufficient, even with long flow, to reverse the P.D.) Records a, b, and c show recovery of positive P.D. on cessation of current flow, the more slowly the lower the P.D. has been carried. Only when it is reduced to about zero, however, as in Record d, does reversal occur, and here it goes on to completion, after a temporary recovery (depolarization), even after the current ceases to flow. Similar depolarizations occur in Records e, f, and g, when the current is interrupted after producing negativity (in Record e, the current is again passed briefly a second time, producing the cusp characteristic of reversal). In Record g the current density is increased to $125 \mu\text{A/cm}^2$, producing a more rapid reversal. After Records d, e, f an inward current of sufficient density was passed (as in Record g) to restore positivity before the next outward flow.

Zero is shown by the longer mark at the beginning of each record and from time to time during the record. Sensitivity about 10 mV per horizontal division indicated by a calibration of 100 mV on Record a and by 50 mV — and — marks inserted at the beginning of each record. Time marks 1 second apart. Residual current (r) outward $0.35 \mu\text{A}$ or less when P.D. is positive, about $0.1 \mu\text{A/cm}^2$ inward when P.D. is negative. Designations as in Fig. 1.

delicately metastable system, but it gives beautiful confirmation of the all or none nature of the reversal and recovery process in this case.

Finally, at sufficiently high ammonia concentrations (e.g., 0.003 to 0.005 M NH_4Cl in sea water at pH 8.0), a threshold is reached at which ammonia alone is able to initiate the reversed potential and maintain it, even after long inward current flows. An example is shown in Fig. 14.

DISCUSSION

The effects of current flow on the P.D. of *Halicystis*, while somewhat complex, may apparently be divided into two types of response, much as in *Valonia*⁴. These are (1) regular polarization produced by small currents in either direction while the P.D. is still positive (and to some extent when it has become negative), (2) the reversal of P.D. or recovery therefrom which is abrupt and characterized by a definite threshold.

While it is, of course, possible that different mechanisms are involved in the two responses, it is helpful to proceed on the assumption that the same changes, produced by current flow, underlie both, the abrupt reversal occurring when the "regular" effect has proceeded beyond a certain point.

One of the effects which might result from current flow across a phase boundary, such as the surface of protoplasm probably presents, is to set up a new concentration gradient of ions due to the greater mobility of ions of one charge than of another through the surface. Models for such polarizations are not numerous, but the effects have been demonstrated in several cases. This new gradient would persist as long as the current flow continued, reaching a steady state in which the backward motion of the ions just balanced their tendency to separate under the potential drop across the surface. Any pair of ions, oppositely charged, could be assumed so to separate. The length of time required for them to migrate to their new positions (or to come back to their original distribution) would represent the time for polarization or depolarization to be completed, the charge tending to pull them back would be the degree of polarization or polarization potential. When the ionic concentration on one side or the other of the membrane or surface reached a critical value, special effects might

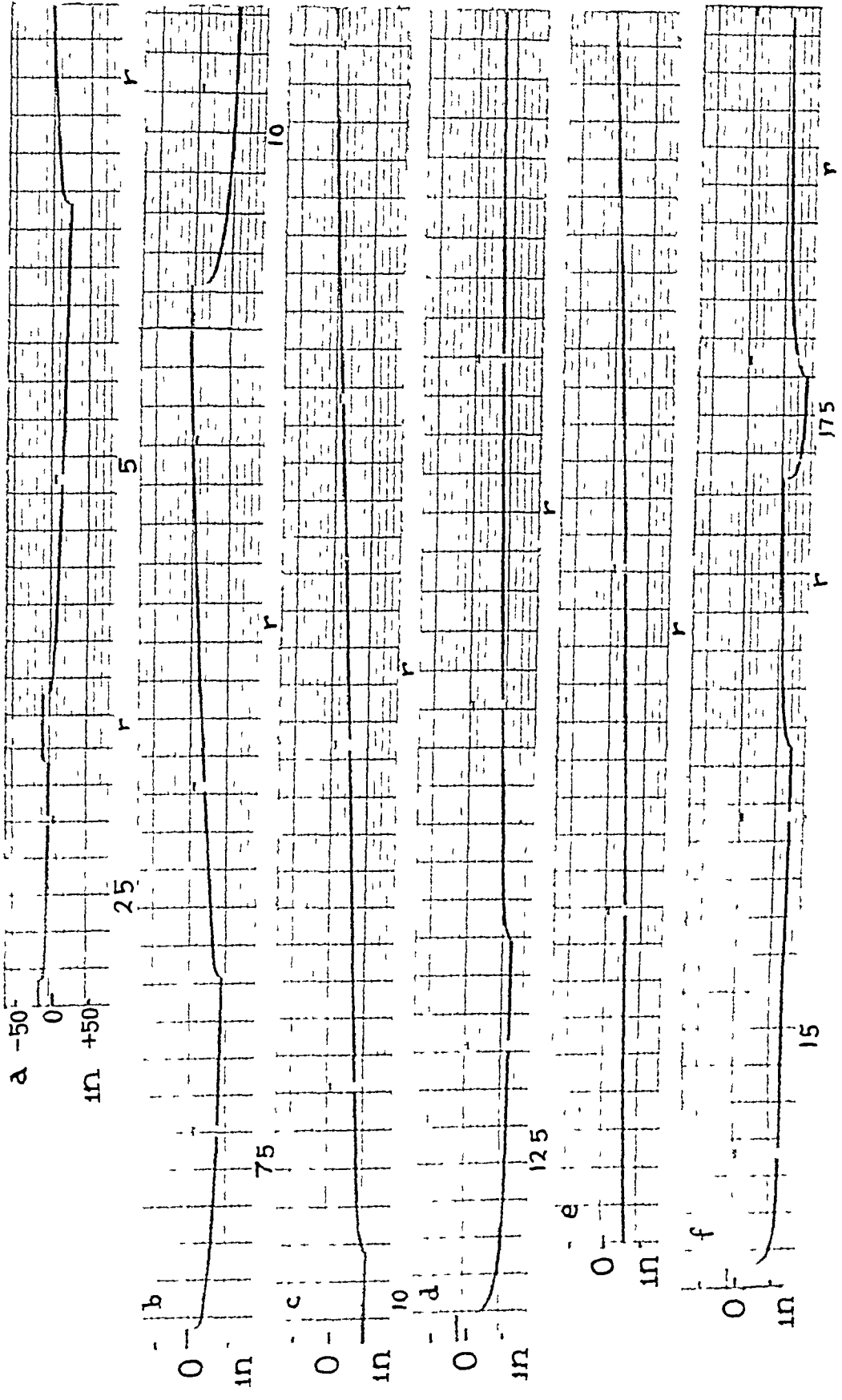


FIG 13

then be produced (the reversal of P D) What evidence have we regarding the kinds of ions which could do this in *Halicystis*?

Previous studies^{2, 5} have shown that both K^+ and H^+ have a pronounced effect on the P D in *Halicystis Osterhoutii* interpretable on the basis of greater mobility for the positive ions than for Cl^- On the other hand dilution of sea water (unpublished results) has an effect which can be interpreted as a greater mobility for Cl^- than for Na^+ much as in *Valonia*⁶ Several possibilities for differential mobility thus exist

Hydrogen Ion—Since we have the most evidence concerning hydrogen ion, this will be taken up first On the basis of the experiments just mentioned it has a relatively high mobility through the outer surface hence would presumably become concentrated at the side where the positive current was leaving With inward currents then, the inside of the protoplasmic surface would become more acid, the outside more alkaline and the effects of inward current flow do indeed resemble those of increased alkalinity on the outer surface (of *H Osterhoutii*), giving a somewhat increased positive P D Conversely, with outward current, the outside would become more acid, and here again the effects are the same as with acidified sea water—a decrease of positive P D Of even more importance, however, the inner side would become more alkaline during outward flow of current, the experiments with ammonia penetration,¹ and with vacuolar perfusion both indicate that when the sap (and presumably the protoplasm also) reached a critical degree of alkalinity, the P D abruptly reversed in sign This may also be what happens at critical outward current densities Finally if the internal reaction of the protoplasm has

FIG 13 Effects of inward current flow on P D of *H Osterhoutii* already made negative (by current flow) in the presence of higher ammonia concentration (0.002 M NH_4Cl in sea water of pH 8.0) Inward currents of increasing density restore positive P D which however becomes negative again on cessation of current The reversal is slower and slower after increasing inward currents until finally a density is reached (15 to 17.5 $\mu A/cm$) which permanently restores positivity Sensitivity 10 mv per horizontal division zero and 50 mv + and - being indicated on each record Time marks 1 second apart Residual current (r) less than 0.35 $\mu A/cm^2$ outward or 0.1 μA inward depending on sign of P D Designations as in Fig 1

⁶ Damon E B *J Gen Physiol* 1932-33 16 375

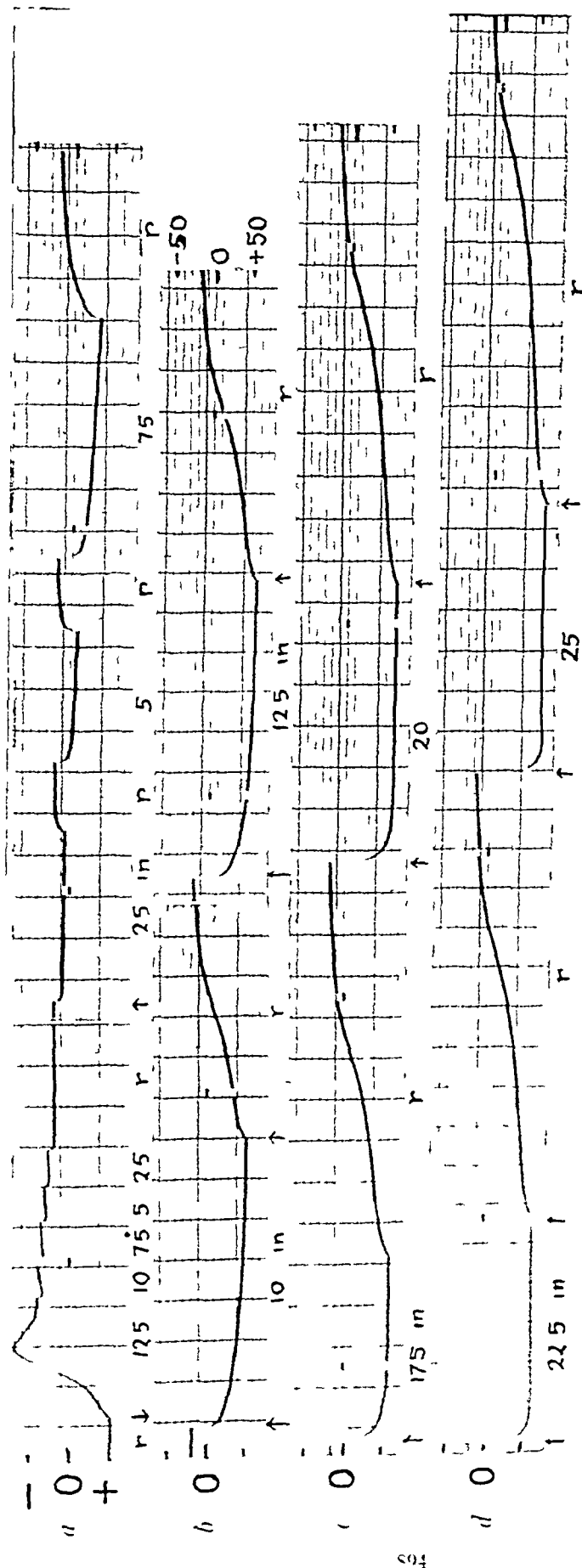


Fig. 11 Effects of current flow on bioelectric potential of *Osterhoutia* in the presence of threshold ammonia concentration (0.003 M NH_4Cl in sea water at pH 8.0). The record was begun immediately after exposure of the cell to this solution. (At this concentration reversal occurs spontaneously in 10 to 15 minutes, but it occurred immediately with $12.5 \mu\text{A}$ flowing outward across the protoplasm.) The outward current was then reduced in equal steps down to the residual current (r), the p.d. remaining reversed. In an effort to produce recovery, inward currents were then passed, successively larger up to $25 \mu\text{A cm}^2$, and while the larger ones restored positivity as long as they flowed, negativity was regained on their cessation, although more slowly after larger currents. Sensitivity 10 mV per horizontal division, zero and 50 mV + and - being marked on Record b. Time marks 1 second apart. Residual current (r) inward or outward, depending on the sign of the p.d., was not over $0.35 \mu\text{A/cm}^2$ and usually less than $0.25 \mu\text{A/cm}^2$. Designations as in Fig. 1. Automatic zero every 9 seconds.

already been rendered alkaline by ammonia, an inward current may cause recovery of positive P.D. by increasing the acidity beyond the critical point.

Leaving entirely out of account the manner in which such change of pH affects the potential (a problem involving the origin of the protoplasmic potential itself) this explanation is reasonably consistent and in harmony with the known facts, at least as regards one species (*H. Osterhoutii*). There are, however, several objections to it, which have been considered in connection with *Valonia*.

An objection to the explanation of current flow by pH effects was found when the California species of *Hyalocystis* *H. ovalis*, was studied from this point of view. Not only were increases of pH in the vacuole produced by perfusion, incapable of producing reversal of P.D. as in *H. Osterhoutii*, but there were no transient effects of acid and alkaline sea water applied externally, which could be interpreted as due to the higher mobility of H ion. Therefore both the theoretical basis for expecting alkalinity by current flow and the actual production of reversed P.D. by alkalinity in the vacuole were absent. Yet P.D. reversal is produced in this species by outward current flow just as in *H. Osterhoutii*, and ammonia is likewise effective although at an appreciably higher threshold for both treatments.

It may be that the locus of the effects lies too deep within the protoplasm, and the two surfaces of the protoplasm are too impermeable to hydrogen ion in this species to be affected by changes of pH in either sea water or vacuole. Yet this very impermeability removes the theoretical basis for a change of pH due to current flow. It is therefore necessary to conclude that although the effects produced by current flow are very similar indeed to those produced by ammonia and that the latter may in turn be explained, at least in one species by an increased alkalinity somewhere in the protoplasm, current flow does not necessarily operate through a change of pH. We may pass on to considering how else it might operate.

Reversal by Other Agents—There is one further treatment now known which can reverse the P.D. without invoking pH changes. This consists of unbalanced NaCl isotonic with sea water.³ The effect is not as reproducible and invariable as ammonia and current flow, but occurs sufficiently frequently to call into question the necessity of pH change

as an essential to reversal. It seems reasonable to suppose that such NaCl treatment increases the permeability of the protoplasm, presumably first at its outer surface, in the well known manner of unbalanced solutions. With its selective ion permeability thus destroyed, the potential existing across this surface (whatever its origin) would now be lost, leaving that at the inner or vacuolar surface to manifest itself for a time alone, until it too was reached by the unbalanced solution. The current flow might produce the same effects by producing a concentration gradient of Na (or K) ions across the surface.

This picture involving the individual integrity of the two surfaces and the potentials across them, would largely account for the two potential levels found so consistently with a great variety of treatments, and the essentially all-or-none nature of their destruction or restoration. Each would be capable of small variations, in response to current flow, ammonia, pH change, etc., but the large variations would be due to the existence or non-existence of the surface itself. By assigning proper values to the individual potentials, the algebraic sum would represent the normal P D, while one alone would give the reversed P D. On this basis, that of the outer surface could be from 150 to 160 mv outwardly directed, and that of the vacuolar surface about 80 mv inwardly directed³. That polarizations can still occur when the P D is reversed shows that one surface, presumably the inner, is still functional.

In what manner, chemical or physical, these layers are affected by the various treatments (ammonia, pH, unbalanced NaCl, outward current) and how inward current can counteract the effect of ammonia must for the present be unanswered. While the pH change still remains the most attractive hypothesis, there are many objections to its full acceptance. It will be discussed again in a subsequent paper in connection with *Nitella*.

Note Added to Proof—It has recently proved possible to control markedly both the protoplasmic potential and the polarizability of *H. ovalis* by various agents influencing metabolism, notably oxygen and light. These render it still more probable that many of the bioelectric effects are due to organic ions produced in metabolism. The effects will be described elsewhere.

SUMMARY

The effect of direct current, of controlled direction and density, across the protoplasm of impaled cells of *Halicystis*, is described. Inward currents slightly increase the already positive P.D. (70 to 80 mv) in a regular polarization curve, which depolarizes equally smoothly when the current is stopped. Outward currents of low density produce similar curves in the opposite direction, decreasing the positive P.D. by some 10 or 20 mv with recovery on cessation of flow. Above a critical density of outward current however, a new effect becomes superimposed, an abrupt reversal of the P.D. which now becomes 30 to 60 mv negative.

The reversal curve has a characteristic shape: the original polarization passes into a sigmoid reversal curve, with an abrupt cusp usually following reversal, and an irregular negative value remaining as long as the current flows. Further increases of outward current each produce a small initial cusp, but do not greatly increase the negative P.D. If the current is decreased there occurs a threshold current density at which the positive P.D. is again recovered, although the outward current continues to flow. This current density (giving positivity) is characteristically less than that required to produce reversal originally, giving the process a hysteretic character.

The recovery is more rapid the smaller the current, and takes only a few seconds in the absence of current flow, its course being in a smooth curve usually without an inflection, thus differing from the S shaped reversal curve.

The reversal produced by outward current flow is compared with that produced by treatment with ammonia. Many formal resemblances suggest that the same mechanism may be involved. Current flow was therefore studied in conjunction with ammonia treatment. Ammonia concentrations below the threshold for reversal were found to lower the threshold for outward currents. Subthreshold ammonia concentrations, just too low to produce reversal alone, produced permanent reversal when assisted by a short flow of very small outward currents, the P.D. remaining reversed when the current was stopped.

Further increases of outward current when the P.D. had been already

reversed by ammonia, produced only small further increases of negativity. This shows that the two treatments are of equivalent effect, and mutually assist in producing a given effect, but are not additive in the sense of being superimposable to produce a greater effect than either could produce by itself.

Since ammonia increases the alkalinity of the sap, and presumably of the protoplasm, when it penetrates, it is possible that the reversal of P D by current flow is also due to change of pH. The evidence for increased alkalinity or acidity due to current flow across phase boundaries or membranes is discussed. While an attractive hypothesis, it meets difficulties in *H. ovalis* where such pH changes are both theoretically questionable and practically ineffective in reversing the P D.

It seems best at the present time to assign the reversal of P D to the alteration or destruction of one surface layer of the protoplasm, with reduction or loss of its potential, leaving that at the other surface still intact and manifesting its oppositely directed potential more or less completely. The location of these surfaces is only conjectural, but some evidence indicates that it is the outer surface which is so altered, and reconstructed on recovery of positive P D. This agrees with the essentially all-or-none character of the reversal. The various treatments which cause reversal may act in quite different ways upon the surface.

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THE KINETICS OF SAPONIFICATION OF IODOACETIC ACID BY SODIUM HYDROXIDE AND BY CERTAIN ALKALINE BUFFER SOLUTIONS*

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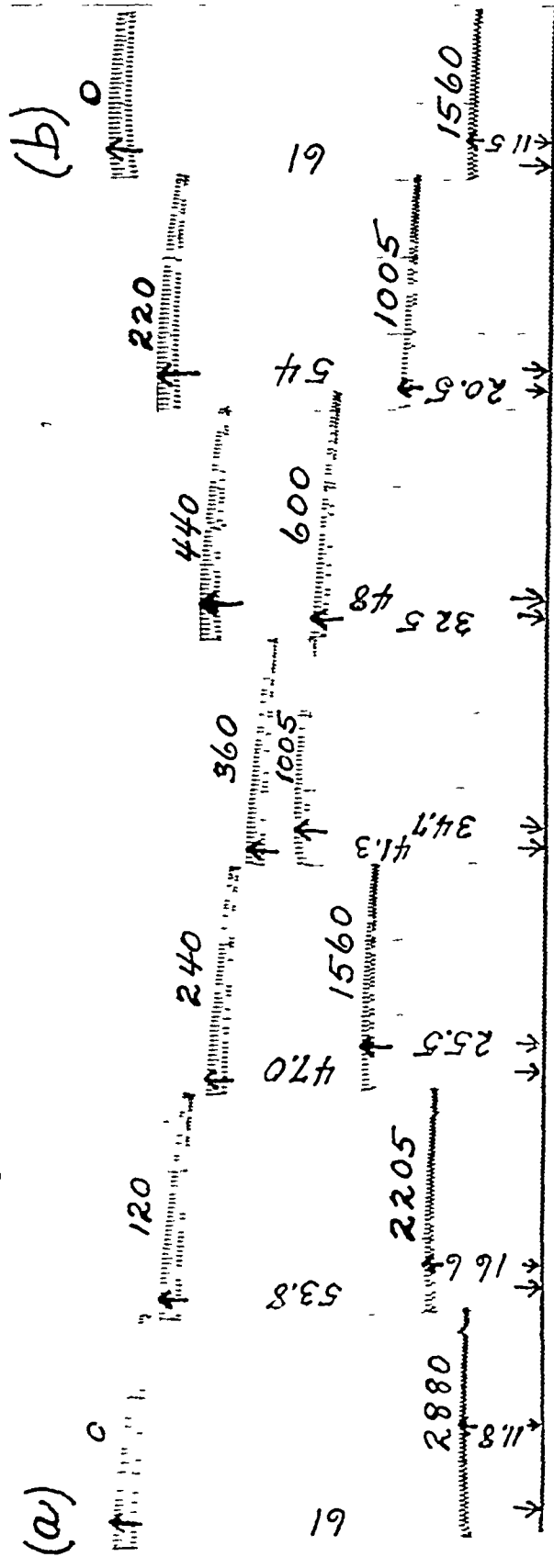
(Accepted for publication October 2 1935)

In studying the reaction which takes place between glycine and iodoacetic acid in certain alkaline buffer solutions (1), it was necessary to consider the saponification of iodoacetic acid by hydroxyl ions. This occurs simultaneously as an independent side reaction. By determining the velocity constants for the saponification in the solutions in which glycine was omitted the rate of the iodoacetic acid-glycine reaction could be obtained. The study of the kinetics of the saponification reaction of iodoacetic acid forms the subject of the present paper. Holmberg (2) investigated this reaction over a limited range of concentration of the reacting constituents. His measurements have been extended in the present paper to regions of greater alkalinity. His paper appeared before the publication of Bronsted's (3) hypothesis, and hence his data were not interpreted on this basis.

The technique employed is essentially the same as that which was described in the previous paper (1). Certain portions of the iodoacetate and hydroxyl ion containing solution which was kept in a thermostat at $25^{\circ} \pm 0.01$ were withdrawn during different time intervals during the saponification. In order to stop the reaction they were brought approximately to neutrality by the addition of HCl. The quantitative estimation of the iodoacetic acid in the samples consisted in recording polarographically the horizontal portions of the current-voltage curves. The concentration of iodoacetic acid can be read from these (see Fig. 1).

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† International Education Board Fellow.



Since the saponification of the iodoacetic acid by OH ions yielding glycollic acid is an ionic reaction which involves salt effects, it was considered desirable to investigate first the reaction rate using sodium hydroxide instead of the buffer solution. In this case, according to Bronsted's hypothesis (3), only the primary salt effect takes place and a positive salt catalysis for this ionic type is to be expected. This reaction is not complicated by side reactions and the ionic strength of the solution remains unchanged during the reaction.

Bronsted's kinetic equation for this type of reaction has the form,

$$-\frac{dx}{dt} = k_0(a-x)(b-x) \frac{f(IAc) f_{OH^-}}{f_{x^-}} \quad (1)$$

in which k_0 is the velocity constant independent of salt concentration, a and b are the initial equivalent concentrations of iodoacetate and hydroxyl ions, respectively, x is the decrease in the concentration of reactants at the time t , and $f(IAc)$, f_{OH^-} , and f_{x^-} are the activity coefficients of iodoacetate, hydroxyl ions, and Bronsted's critical complex, respectively. The values for the activity coefficients can be calculated theoretically only in extremely dilute solutions, for which the Debye-Hückel limiting laws (4) can be applied, but they can be determined also in higher concentrations empirically from solubility measurements or by other thermodynamical methods (5). The estimation of the value for f_{x^-} is doubtful since the existence of the critical complex is an hypothetical one. The velocity constant, k , calculated on the basis of the classical bimolecular kinetic equation,

$$k = \frac{2.303}{t(b-a)} \log \frac{a}{b} \frac{(b-x)}{(a-x)} \quad (2)$$

involves the factor $\frac{f_{IA^-} f_{OH^-}}{f_{x^-}}$ in Bronsted's kinetic equation (1) which used to be denoted simply by F . This factor is expressed by the relationship,

$$k = k_0 F \quad (3)$$

Knowing the value for F at one ionic strength, for which the value for f_{x^-} was assumed to be equal to a known value of the activity coefficient of a divalent anion at the same ionic strength, other values

for F can be calculated by means of equation (3) when k is determined experimentally

Table I demonstrates the calculation of the bimolecular velocity constants according to equation (2) for the initial concentrations

(a) $a = 0.0198$ N sodium iodoacetate
 $b = 0.310$ N sodium hydroxide

(b) $a = 0.0198$ N sodium iodoacetate
 $b = 0.520$ N sodium hydroxide

Since the values for $(a - x)$ represent the reading of the current intensities in millimeters, (which are proportional to the concentrations

TABLE I

(a) NaOH = 0.310 N				(b) NaOH = 0.520 N			
Time	$(a - x)$	$(b - x)$	$k \times 10^4$	Time	$(a - x)$	$(b - x)$	$k \times 10^4$
min				min			
0	61	961.2		0	61	1602	
220	54	954.2	18.0	120	53.8	1595	20.2
440	47.7	947.9	18.2	240	47	1588	21.0
1005	34.7	934.9	18.3	360	41.3	1582	21.0
1560	25.5	925.7	18.3	600	32.5	1574	20.4
2205	16.6	916.8	18.3	1005	20.5	1562	21.2
2880	11.8	912.0	18.7	1560	11.5	1553	21.0

Na(I CH₂COO) = 0.0198 N

of iodoacetic acid (see Fig 1)), the values of b must be expressed in the same arbitrary units, *viz*, they must be multiplied in this case by the factor 61/0.0198. The values for $(b - a)$, however, must be expressed in equivalent concentrations

Table II shows the values for the velocity constants, k , obtained from experiments in which the initial concentration of iodoacetic acid was kept constant ($a = 0.0198$ N), while the initial concentration of sodium hydroxide was gradually increased

The observed increase of the values of k with the increased concentration of sodium hydroxide must be related to the increase of the factor F , which depends upon the ionic strength. In Fig 2 the velocity constants, k , are plotted against the ionic strength, μ . Accepting

for F in a solution of ionic strength, $\mu = 0.1$, the value of 1.22, which is given empirically from thermodynamical measurements by La Mer (6), the value, k_0 , can be calculated on the basis of equation (3) and, in this case, is equal to $\frac{14 \times 10^{-4}}{1.22} = 11.5 \times 10^{-4}$. The values for F corresponding to resulting ionic strengths are determined by the ratios k/k_0 . This factor, F , accounts for the primary salt effect, showing a distinct positive salt catalysis. It is worth mentioning that the curve

TABLE II

NaOH normality	$k \times 10^4$	μ	$F = \frac{k}{k_0}$
0.0414	13.0	0.06	1.13
0.083	14.0*	0.103	1.22
0.109	14.7	0.129	1.28
0.310	18.3	0.330	1.59
0.520	21.0	0.540	1.83
0.700	23.3	0.720	2.03
0.873	24.6	0.893	2.14
1.000	26.0	1.020	2.26
1.120	27.7	1.140	2.41
1.36	30.9	1.380	2.69
1.60	35.0	1.62	3.04
2.00	43.0	2.02	3.74
2.40	49.4	2.42	4.30
2.67	52.4	2.69	4.56

*Holmberg (2) found for 0.05 N NaOH + 0.05 N Na(I CH₂COO) $10^4 \times k = 13.9$

in Fig. 2 shows an inflex point at an ionic strength in the neighborhood of 0.8 μ , at which the activity coefficients of alkali hydroxides show minimal values (7).

The positive salt catalysis could be demonstrated also qualitatively by an increased rate of saponification when neutral salts were added to the reacting medium. Thus the presence of 0.5 N KCl in 0.709 N NaOH and 0.0198 N Na(I CH₂COO) increases the velocity constant from the value of 14.7×10^{-4} to a value of 23.5×10^{-4} , and the presence of 0.6 N Na₂SO₄ in 0.083 N NaOH and 0.0198 N Na(I CH₂COO) from the value of 14×10^{-4} to a value of 23.5×10^{-4} .

The measurements of the velocity constants of the saponification of

iodoacetic acid in buffer solutions were carried out by using the same experimental technique. In the set of experiments which are given in Table III, 0.247 M boric acid in 0.1 N KCl, mixed with varying concentrations of NaOH, was used. The pH was determined by means of a standardized hydrogen electrode. The titration curve of 0.247 M boric acid is known (1). For this set of experiments the concentration of iodoacetate was 0.0198 N. The velocity constants,

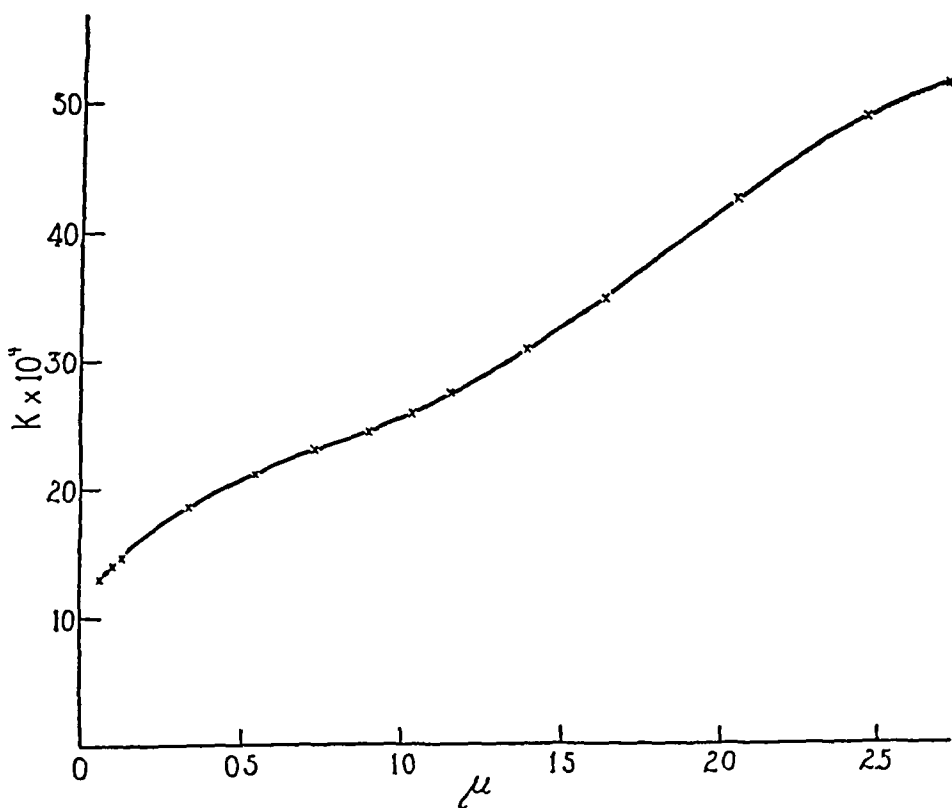


FIG. 2 The relation of the bimolecular velocity constant of the saponification of iodoacetate to the ionic strength of the solution

denoted by k_s , and related to the given pH, were calculated by using the formula for monomolecular reactions,

$$k_s = \frac{2.303}{t} \log \frac{a}{a-x}, \quad (4)$$

in which a represents the initial concentration of iodoacetate and x its decrease at the time t . The use of this equation was found satis-

factory insofar as the decrease of hydroxyl ions during the reaction did not appreciably affect the pH. At lower values of pH a decrease of k , was observed, which, nevertheless, permitted its rough estimation. In order to obtain the bimolecular constants which could be compared with the corresponding values of k in Table II, the values of k , must be divided by the concentration of hydroxyl ions. By determining the pH of the reacting medium, the activity of the hydroxyl ions, ($a_{\text{OH}^-} = f_{\text{OH}^-} (\text{OH}^-)$), instead of their concentration, can be calculated. If the values of k , are divided by the activity of OH^- ions, the values of $\frac{k}{f_{\text{OH}}}$ are obtained. A comparison between the values of $\frac{k}{f_{\text{OH}}}$ and k at the same ionic strength in the first three lines of Table III conforms approximately with the value for

TABLE III

pH	NaOH normality	$k \times 10^4$	$f_{\text{OH}} \times \text{OH}$	$\frac{k \times 10^4}{f_{\text{OH}} \times \text{OH}}$	$k \times 10^4$	μ
12.96	0.415	3.7	0.091	40	21	0.535
12.49	0.311	1.1	0.031	35	20	0.431
12.00	0.269	0.30	0.010	30	19.5	0.389
11.20	0.249	0.10	0.0016	63	19	0.369
9.91	0.208	0.08	0.00008	984	18.3	0.328

f_{OH^-} which, in this case, should be expected to be about 0.6—0.7. The values for k , at pH = 11.2 and 9.91, on the other hand, do not show any proportionality to the concentration of hydroxyl ions, rather, their proportionality to the concentration of sodium hydroxide or borate anion in the buffer solution seems to be satisfied. This relationship which holds in the pH range below the neutralization of boric acid might be related to the principle of general basic catalysis (8). This principle involves the cases in which the velocity constants are proportional to the anion of the weak acid which, in the sense of Bronsted's interpretation, is to be regarded as a base. In order to apply this principle to the described problem, more detailed experiments must be made.

The author is indebted to Professor Carl L. A. Schmidt for his helpful interest during the work.

SUMMARY

1 The rate of the saponification of iodoacetic acid in sodium hydroxide and alkaline buffer solutions yielding glycollic acid was measured by means of Heyrovský's polarographic method

2 From the bimolecular velocity constants, increasing with the ionic strength of the solution, the Bronsted factor, F , which characterizes the primary salt effect, was calculated

3 In the borate buffer solutions the monomolecular constants of the saponification were determined which, at values above the pH of neutralization of boric acid, show a proportionality to the concentration of hydroxyl anions. Below the pH of neutralization of boric acid, they are proportional to the concentration of borate anions

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THE ADSORPTION OF EGG ALBUMIN ON COLLODION MEMBRANES

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The adsorption of gelatin on collodion membranes as a function of pH and of protein concentration has been studied by Hitchcock (6, 7) and by Palmer (13). Hitchcock showed that as the gelatin concentration increased the adsorption followed a hyperbolic curve corresponding to the Langmuir adsorption isotherm, becoming asymptotic to a saturation value, and that the adsorption from dilute solutions was at a maximum near the isoelectric point, decreasing in solutions on either the acid or the alkaline side. Palmer showed that when membranes of low permeability were used the saturation levels varied with the pH, being highest at the isoelectric point and lower on either side. This difference was abolished by the addition of 0.4 M NaCl to the solutions. These results were shown to be consistent with Loeb's theory that gelatin exists in solution in the form of submicroscopic particles capable of imbibing water and swelling according to the Donnan theory of membrane equilibria.

Hitchcock showed that the adsorption of egg albumin from dilute solutions on collodion membranes varied with the pH in the same way as did that of gelatin. Other properties of egg albumin solutions, however, particularly the viscosity, do not support the view that the units of this protein dispersed in water are capable of any great degree of swelling. The present investigation was laid out along the same lines as that of Palmer in order to find out whether egg albumin would behave in the same way as gelatin and whether the saturation levels of adsorption might here too be taken as an index of particle size and of variations in it, if any.

*From a dissertation presented to the faculty of the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, May, 1935.

EXPERIMENTAL METHODS

Preparation of Egg Albumin Solutions—The egg albumin was prepared by a method developed by Cannan (2). After removal of the globulin, the albumin was precipitated in crystalline form by the addition of anhydrous sodium sulfate at pH 4.6. All preparations were thus crystallized three times. Solutions of the purified protein were dialyzed against flowing, slightly acidified distilled water in stoppered cellophane tubes until their conductivity fell to 1 or 2×10^{-4} mhos. For work at high protein concentrations, water was then removed by hanging cellophane tubes containing the solution in front of a fan, according to Kober (8). In this way concentrations as high as 24 per cent could be obtained.

Such stock solutions of egg albumin were brought to the required pH by the addition of 0.2 N hydrochloric acid or sodium hydroxide, and the desired concentrations were obtained by dilution with hydrochloric acid of the same pH as that of the acid, alkaline, or isoelectric protein solution.

Preparation of Collodion Membranes—To prepare the collodion membranes, Merck's collodion U S P X was diluted with an equal volume of a mixture (3:1 by volume) of ether and 95 per cent ethyl alcohol. To each 100 cc. of this dilute collodion was added 3.5 to 4.0 cc. of ethylene glycol, following the method of Pierce (14). About 70 cc. of this mixture was poured on a mercury surface about 38 cm. in diameter and the solvents were allowed to evaporate. At first an attempt was made to dry these membranes in a closed chamber with a stream of dried air, but in the small chamber used it was found that the air current necessary to remove so large a volume of alcohol and ether in a day was too rapid to permit the formation of a uniform membrane. Most of the membranes used, therefore, were allowed to dry in the laboratory air (temperature about 25°C. and relative humidity about 25 to 30 per cent), protected merely by a gauze-covered cylindrical metal shield. With this method there was a slight concentric variation due to evaporation during spreading, which was taken into account in the calculations.

Each membrane so prepared was removed from the mercury without wetting, and with a steel die 37 disks 3.81 cm. in diameter were cut from it, in concentric rings. These disks were then washed at least a day in several changes of distilled water before use. Each disk, after drying at 110°C., weighed usually about 22 mg.; those from different rings might vary as much as 1 mg., but those from a single ring not more than 0.2 mg. between themselves. In calculating adsorption, each disk was always compared with a blank from the same ring. Different membranes from day to day might vary considerably in permeability and dry weight, but each complete experiment was run with disks from a single membrane. The permeability was not determined for each membrane used, but this was done for a great many test membranes. The flow of water of known temperature through a fixed area of a disk under known pressure was timed, the thickness of the disk was measured with a screw micrometer, and the results were calculated by the Manegold formula (11) for specific permeability. For the membranes which gave the best results the specific permeability was of the order of 1 to 2×10^{-14} sq. cm.

Adsorption Experiments—The adsorption experiments were performed in a manner quite similar to that of Hitchcock and of Palmer. Three collodion disks were placed in each of a series of 50 cc. wide mouth Florence flasks containing 20 to 30 cc. of egg albumin solution. These flasks were stoppered with paraffined corks and clamped on a rocking bar in a water bath at $25.0^{\circ}\text{C} \pm 0.03^{\circ}$ and rocked gently at about 60 cycles per minute for 18 to 22 hours. After this time the disks were taken out, rinsed 3 minutes in each of four changes of distilled water, dried in a 110° oven for at least 2 hours, and weighed to the nearest 0.1 mg. The weights obtained were compared with the weights of similar disks not treated with protein and the adsorbed albumin was determined by difference. On account of the slight variation in the disks the adsorption figure was divided by the weight of the blank, and the values were thus reduced to a common unit, milligrams of protein adsorbed per milligram of dry collodion.

After the disks were removed, the pH of each solution was determined with the DuBois pipette type glass electrode (4, 5) and DuBois vacuum tube potentiometer (3). The concentration of protein was determined by the use of a dipping refractometer. On the basis of a standardization of this instrument against the dry weight of 5 cc. samples of test solutions, the factor 1.82×10^{-3} was used to convert the increment of refractive index to protein concentration in grams per 100 cc.

EXPERIMENTAL RESULTS¹

With two exceptions, to be noted later, the results of Hitchcock (6, 7) on the adsorption of egg albumin have been confirmed. When the amounts adsorbed from solutions of the isoelectric protein are plotted against the protein concentration, the points can generally be fitted by a curve of the Langmuir type, a rectangular hyperbola rising steeply from the origin and becoming horizontal at a height representing saturation of the membrane. This level has been found to be, as in the case of gelatin, a function of the permeability of the membranes, rising rapidly with increasing permeability. The membranes used were designed to be the most permeable ones which would become saturated at protein concentrations between 5 and 7 per cent.

A great many experiments of the type reported by Palmer were performed, and typical results are given in Fig. 1. As shown by the curves, the apparent saturation level is lower when the pH of the solutions is removed in either direction from the isoelectric point. This difference is not present if the solutions of acid or alkaline protein contain 0.4 M NaCl. Control experiments have shown that the

¹Since the dissertation upon which this paper is based included some fifty out of the eighty complete experiments run, only representative experiments are reported here. Complete data, in tables, may be found in the dissertation.

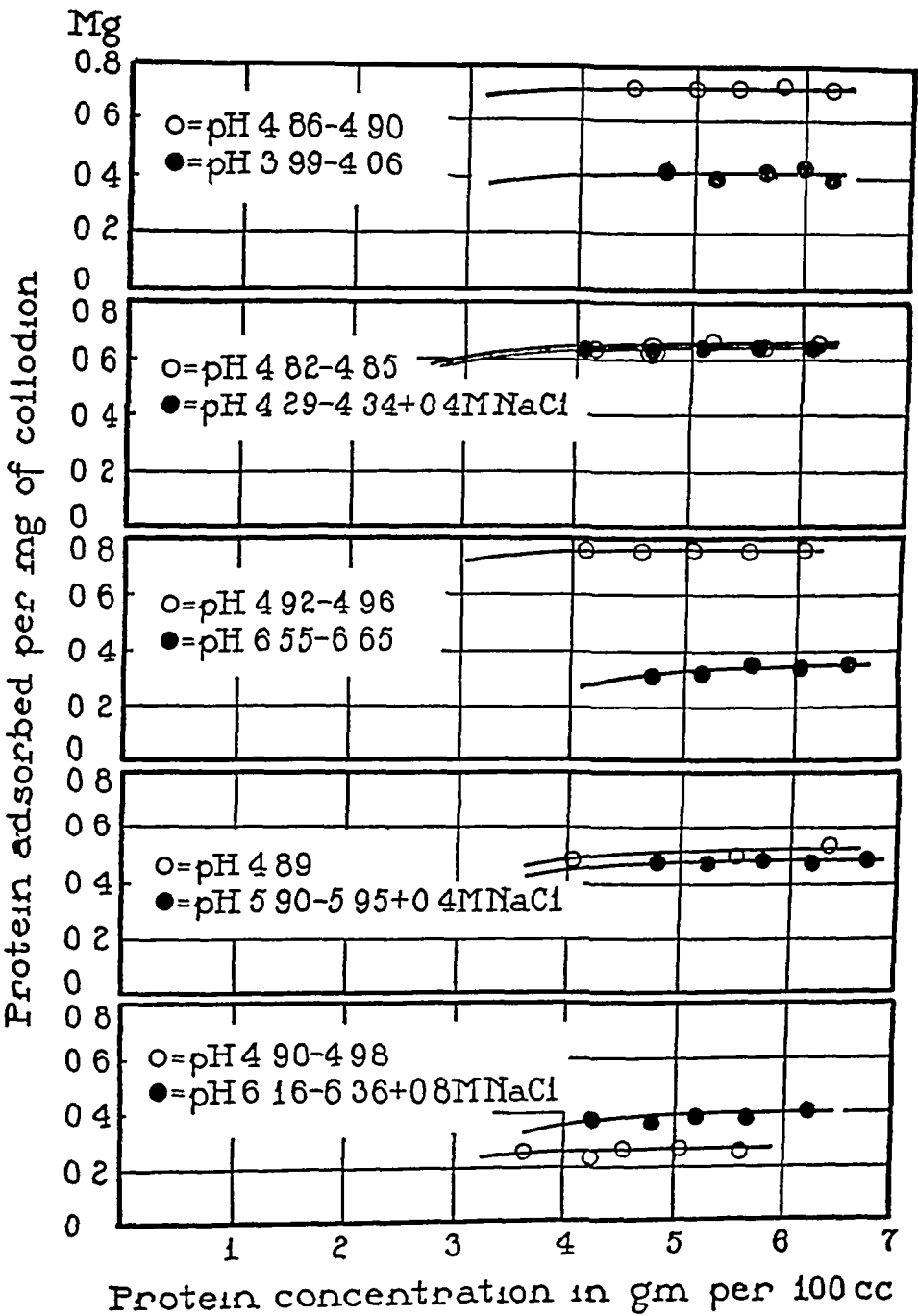


FIG 1 Adsorption of isoelectric, acid, and alkaline egg albumin, with and without salt

adsorption from solutions at the isoelectric point is not influenced by the addition of salt

Two departures from Hitchcock's results have been noted in these experiments. When the range of protein concentration is extended to 24 per cent, it is consistently found that a break in the curve occurs somewhere between 5 and 12 per cent. The earlier points, representing the more dilute solutions, can be fitted by one curve, the later points, for the concentrated solutions, by another, no smooth, continuous curve will do for both. This has been indicated in Fig 2 by the dotted section in the curve for the adsorption of isoelectric protein. When more points were obtained in such experiments, the breaks were more pronounced.

The second difference between these results and those of Hitchcock lies in the location of the pH of maximum adsorption. This has been consistently found to be at 5.0, as shown by the first curve of Fig 3. Even allowing for the discordant findings of various workers as to the isoelectric point of this protein, and for a considerable variation of it under different conditions, the pH of maximum adsorption would seem still to be significantly on the alkaline side of the isoelectric pH.

A comparison of the curves of Fig 1 (with the exception of the last one) with those reported by Palmer would suggest a marked similarity between the adsorption of egg albumin and that of gelatin. The results presented in Fig 2, however, show that the case is not so simple. Whereas Palmer's curves for gelatin seem to be all of the same type, differing only in the saturation level, those for egg albumin are seen to change markedly in shape as the pH of the solutions recedes from the isoelectric point. The data obtained for the adsorption from the more acid solutions can be represented only by a curve rising at first less steeply than that for the isoelectric protein, passing through one or more apparently level stages, and finally in many cases crossing to reach points above the isoelectric curve. The experiment given in Fig 2 is chosen because it shows three pH regions. The number of points is limited by the number of disks available from a single membrane, but experiments giving more points for fewer pH regions have justified the drawing of the curves as here given.

The curves shown in Fig 3 illustrate a different type of experiment, in which the concentration of protein was kept constant at about 6

per cent while the pH was varied. Salt-free solutions and two levels of salt concentration were studied. The three experiments were necessarily done with different membranes which, due to the weather, happened to vary considerably in permeability. The scales for plotting have therefore been chosen to show comparable relative variations in adsorption. The curve for the salt-free solutions is much like that reported by Hitchcock, except that the grouping of points shows the maximum to be at about pH 5.0 instead of 4.8. The other curves show that the addition of salt to the solutions on the acid side brings the adsorption level close to that at the isoelectric point,

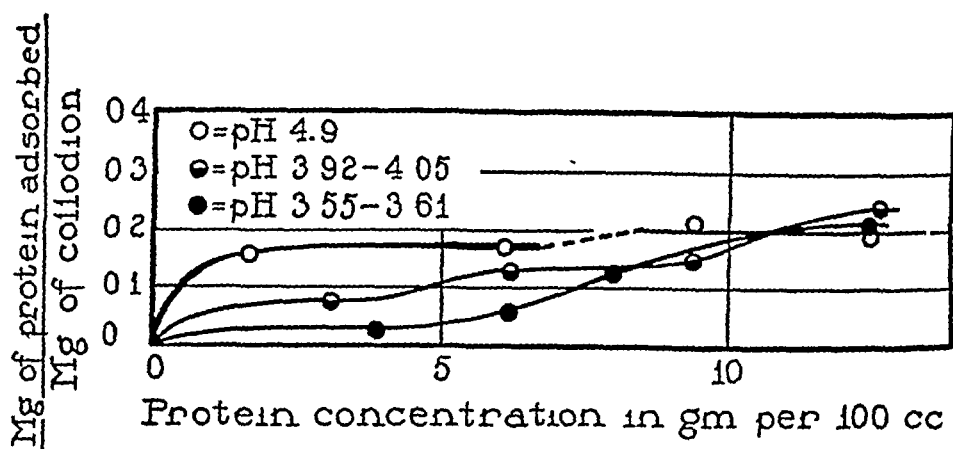


FIG. 2 Adsorption of isoelectric and acid egg albumin

but on the alkaline side the adsorption may be pushed far above this value. This effect is also shown in the last curve of Fig. 1.

Two experiments not shown in the figures have brought out a point which, on further investigation, may prove significant in elucidating the factors involved in the adsorption behavior under consideration. In these experiments one set of solutions was kept as usual in the 25° bath while another set was kept in the cold room at about 1°C. Although the temperature coefficient of most adsorption processes has been found to be negative, in this case the adsorption at 25° was about twice as great as that at 1°.

Some attempts were made to use cellophane instead of collodion as the adsorbing agent, but it was found to take up no protein at all.

At first this was thought to indicate that the chemical differences between the two might be significant, but later it was found that cellophane swollen with alkali until its permeability was comparable

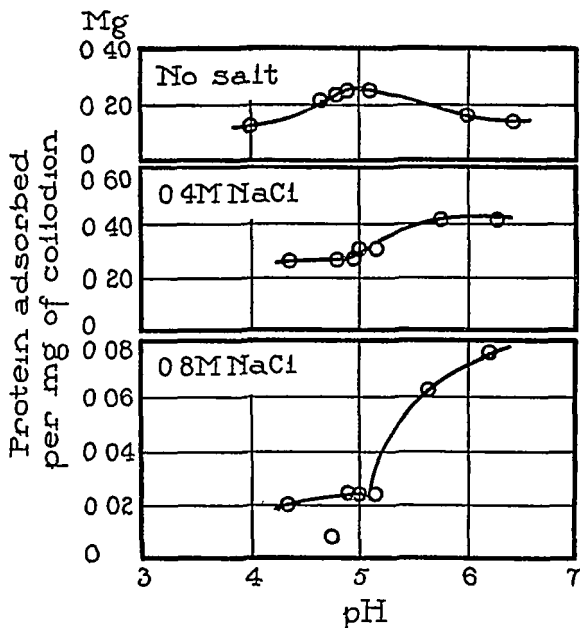


FIG 3 Effect of pH on the adsorption of egg albumin from 6 per cent solutions, with and without salt

with that of the collodion membranes would also take up protein. Cellophane was rejected as an adsorbent, however, because disks cut from it seemed less uniform in dry weight than those from collodion membranes.

DISCUSSION OF RESULTS

To quote from Palmer (13),² the results of his experiments with gelatin "can be explained by, and seem to lend support to, the theory proposed by Loeb and further developed by Kunitz concerning the effects of pH and salt on the size of gelatin particles in solution." In view of the known differences between gelatin and egg albumin, particularly in the dependence of the viscosity of their solutions on pH, it was rather expected that the results of the present investigation might not lend themselves well to such an interpretation. This expectation has been fulfilled up to a certain point the results are quite similar, but some other hypothesis must be brought forward to explain the curves of Fig 2 and Fig 3. As between the two proteins, if the above explanation for the case of gelatin be granted, it is the similarities rather than the differences that must be reconciled.

Since these experiments were done the work of Lindau and Rhodius (10) on the adsorption of egg albumin on quartz powder has appeared. These authors believe that the protein molecules actually change in size with variations in pH, but they ascribe these differences to alterations of intramolecular electrostatic charges, rather than to a Donnan membrane equilibrium. Their theory is similar to that advanced by Tolman and Stearn (15) to account for the swelling of fibrin in acids and alkalies. But the present writer believes that this theory too is not adequate to explain the stepwise curves obtained in these researches or the effect of salt in the solutions on the alkaline side of the isoelectric point. It should be noted, however, that Lindau and Rhodius used protein concentrations of 0.01 to 0.1 per cent, in contrast to the values here reported of 0.1 to 23 per cent. It should also be mentioned that Tolman and Stearn reported that the addition of NaCl to their acid solutions caused a decrease in swelling even below that observed in water alone.

The complete solution to the problem is not to be found in the experiments so far performed. It is proposed to continue the investigation along lines which may throw more light on the problem. Abderhalden and Fodor (1) found, in the case of the adsorption of casein and of a yeast juice protein upon charcoal, a dependence of

adsorption upon pH similar to that observed for gelatin and egg albumin. They believed that the adsorption was a function of the stability of the colloidal system, probably dependent upon the hydration of the suspended particles. This is one hypothesis that seems, in the present case, more useful than that of Loeb and Kunitz.

An alternative hypothesis is that variations in pH and in salt concentration may affect the forces causing the protein to be retained on the membrane. Landsteiner and Uhlirz (9) and Michaelis and Rona (12) came to the conclusion that definitely chemical forces were responsible for the adsorption of proteins on various substances. It is possible that variations in the ionization of egg albumin might alter such forces; the consequent adsorption behavior might then be quite different from that to be expected if the particle size, governed by a Donnan equilibrium, were the only determining factor. The sign of the temperature coefficient noted earlier in this paper might well fit better into this hypothesis, since it is inconsistent with the findings in other adsorption processes.

In view of the high degree of irreversibility of the adsorption of proteins, Michaelis and Rona (12) put forward their belief that adsorbed proteins were denatured. If this should be true, it is not yet possible to say whether only denatured egg albumin is adsorbed, whether it is denatured during the process of adsorption, or whether once adsorbed it is particularly susceptible to denaturation. The adsorption maximum at pH 5.0 may have a bearing upon this question, but in the absence of reliable data on the isoelectric point of denatured egg albumin further researches with this possibility in mind will be necessary before a definite statement can be made.

It is hoped that the key to the usefulness of these hypotheses may lie in a further study of the adsorption from concentrated solutions and of the effect of smaller gradations of salt concentration, possibly of different salts, and in a more thorough investigation of the effect of variations in temperature.

The writer wishes to express his gratitude to Dr. David I. Hitchcock for the suggestion of this research and for his advice and assistance, and to Mr. Delafield DuBois for permission to use his glass electrodes and vacuum tube potentiometer.

SUMMARY

An experimental study has been made of the adsorption of purified egg albumin, from aqueous solution, on collodion membranes. At protein concentrations of 4 to 7 per cent apparent saturation values were obtained which resembled closely the results obtained with gelatin, showing a maximum at pH 5.0 and lower values on either side of this region. Over large ranges of protein concentration, however, the curves for the adsorption from solutions removed in either direction from the isoelectric point exhibited a different shape from the hyperbola obtained in the neighborhood of pH 5.0. The addition of NaCl to solutions on the acid side tended to obliterate the effect of the pH difference, on the alkaline side it greatly increased the adsorption. The adsorption at 25° was about twice as great as that at 1°.

The theory of the swelling of submicroscopic particles, advanced to account for the adsorption behavior of gelatin, is not sufficient to explain the results obtained with egg albumin. It is suggested that the effect is related to alterations in the forces causing the retention of the protein on the membranes.

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IONIC TRANSFERENCE NUMBERS IN CELLOPHANE MEMBRANES

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The object of this paper is to present data concerning the ionic transference numbers, or the relative ionic mobilities, for some univalent chlorides in aqueous solution *within* a cellophane membrane as determined by means of "concentration chains." Originally this investigation was intended to furnish "mobility" ratios for insertion in formulas predicting the effects of interdiffusion upon ionic distribution ("diffusion effect" *cf* (1)). The results, however, offer some additional evidence on the subject of the permeability of membranes, which is of biological interest.

Although the influence of membranes upon diffusion has been mentioned by many earlier workers, a systematic investigation on this subject was first started by Michaelis and his collaborators about 1925 (2), and later, from somewhat different viewpoints, by Manegold (3) and others. As far as electrolytes are concerned, the results of these authors indicate that (porous) membranes exert, in general, a decided influence upon the diffusivity of the ions, attributed, particularly by Michaelis, mainly to the "pore" size and the "charge" of the pore walls within the membrane.

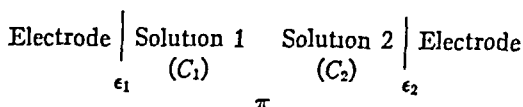
Fujita (4) reported a series of mobility relations obtained by using parchment. These results were obtained in a fashion that can give only a semiquantitative effect. No good data seem, however, to have been published for cellophane, which is a more convenient and reproducible material for diffusion experiments.

Principle and Procedure

The method employed here is, in principle, identical with the one used by Michaelis (2*a*), and outlined in textbooks of electrochemistry.

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(*cf* for instance, Lewis (5)) It consists in measuring the total potential, E , of a "concentration chain with transference,"



The solutions contain only the single electrolyte under investigation in the concentrations C_1 and C_2 . The total E M F of a chain of this type is

$$E = (\epsilon_1 - \epsilon_2) + \pi \quad (1)$$

where ϵ_1 and ϵ_2 denote the potentials at the measuring electrodes employed and π is the "liquid junction" or "diffusion" potential

1 *Calomel Electrodes*— $\text{Hg}/\text{Hg}_2\text{Cl}_2$, KCl , were used by Michaelis *et al*. Due to the symmetrical setup and the generally accepted assumption that a high concentration of KCl abolishes any extra liquid junction potentials, the term $(\epsilon_1 - \epsilon_2)$ in Equation 1 is considered to vanish. In other words, the total E M F is essentially equal to the liquid junction potential, which, for this case, according to Nernst (6) is in millivolts

$$E = \pi = \frac{\frac{u}{n_+} - \frac{v}{n_-}}{\frac{u}{n_+} + \frac{v}{n_-}} 0.05916 T \log \frac{C_1}{C_2} \quad (2)$$

where u and v are the mobilities of cations and anions respectively, n_+ and n_- the valency of these ions, and T is the absolute temperature. Confining the treatment to uni-univalent strong electrolytes, considering the activity correction (γ being the mean activity coefficient), and introducing the *transference number*, t^+ , of the cation

$$t^+ = \frac{u}{u + v} \quad (3)$$

we may write Equation 2 as

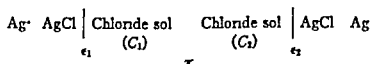
$$t_{\text{calom}}^+ = \frac{1}{2} \left[1 + \frac{E}{0.05916 T \log (C_1 \gamma_1 / C_2 \gamma)} \right] \quad (2a)$$

which is the formula for determining the cation transference number using calomel electrodes. The values of t_{calom}^+ and the mobilities thus calculated are valid for the finite concentration interval measured in the cell and correspond to some concentration intermediate between C_1

and C_2 . A more thorough examination of this formula will be given in the "Discussion" below

2 *Electrodes reversible* in regard to one of the ionic constituents of the solution (for instance, the Ag/AgCl electrode when dealing with chlorides) should be more satisfactory than the calomel electrode, because no "extra" liquid junction potentials are present. In spite of the numerous statements in the literature that a "KCl bridge" abolishes these potentials, the use of it is not theoretically justified as yet. Furthermore there is evidence that, in acid solutions in particular, extra E.M.F.'s still appear (*cf* Bjerrum (7)) of an order which has an appreciable effect upon the computed value of t^+ , sensitive as t^+ is from changes in E as can be read off from Equation 2a. It is true, that the use of, say, silver chloride electrodes introduces a finite value for the term $(\epsilon_1 - \epsilon_2)$ in Equation 1. It does not matter, however, because the final formula to employ for such a case, Equation 4, will contain the same variables as in the calomel electrode case, Equation 2a

The "electrode potential" $(\epsilon_1 - \epsilon_2)$ of a chain of the type

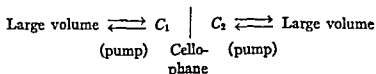


is, as is well known, equal to $0.1983 T \log C_1 \gamma_1 / C_2 \gamma_2$. Adding the liquid junction potential, π , from Equation 2 and again introducing the transference number, leads to

$$t_{\text{AgCl}}^+ = \frac{E}{2 \cdot 0.1983 T \log (C_1 \gamma_1 / C_2 \gamma_2)} \quad (4)$$

This formula may serve for approximate determination of *transference numbers* using, say, *silver chloride* electrodes (*cf* in this connection the paper by Brown and MacInnes (8)). Some further considerations concerning Equation 4 will be deferred to the "Discussion."

The *experimental setup* was as follows. On the opposite sides of a cellophane membrane aqueous solutions of either HCl, KCl, NH_4Cl , NaCl, or LiCl were placed, the concentrations being C_1 and C_2



C_1 and C_2 were maintained constant by continuous renewal from large volumes (1000 cc) and kept homogeneous up to the membrane surface by efficient stirring. The apparatus employed was the one described elsewhere (17). The electrodes were inserted in the "chambers" (of about 30 cc capacity to which "pumps" lead) surrounding the membrane. This was "Cellophane 600" and had a thickness in wet condition of 0.09 mm, its surface covered with solution was about 14 to 15 sq cm, and it showed an average diffusion coefficient¹ for 0.1 to 0.01 N HCl diffusing against a large volume of H₂O of 0.323 in this setup.

The Electrodes—The calomel electrodes were of the type sketched in Fig. 1, having its compact shape in order to allow convenient handling in the apparatus referred to. During the measurement the stop-cock e is open, allowing a very slow flow of saturated KCl through the dense sintered glass plug, r , thus producing a kind of "flowing junction." It gives very reproducible values. The danger of significant contamination with KCl of the solutions into which the electrodes dip is negligible, provided the plugs are dense enough.

The silver chloride electrodes were made strictly according to Brown (9). The different electrodes matched within less than 1 mv. Tested in a concentration chain of KCl, over a range of concentrations extending from 0.1 N to 0.001 N, using a saturated KCl agar "bridge," the electrodes used showed a straight line when plotting log (activity) against EMF. The slope was 58.1 mv. at 22.2° (calculated 58.5 mv.).

The EMF was measured by means of a vacuum tube voltmeter² with a constant "zero." The instrument was used as a direct reading voltmeter using a Leeds and Northrup wall galvanometer Type P with lamp and scale. The setup had a sensitivity of 5 mm. per millivolt. This arrangement draws an extremely small amount of current from the "chain" and could be connected in a closed circuit all the time, thus offering a convenient method of continuous potential control. By means of a D P D T switch the calomel electrode pair and the AgCl electrode pair could be coupled in series with the voltmeter in rapid succession.

The measurements with each particular electrolyte started with the same concentration on both sides. Any potential showing up under this condition was regarded as an "asymmetry potential" of the chain and was used as "blank value" being subtracted from the subsequent readings. The asymmetry was at maximum 2 and 0.5 mv. for the calomel and AgCl electrodes respectively.

¹ The diffusion coefficient, k , is defined by

$$C_t = C_0 e^{-\frac{k}{v}}$$

C_0 = initial concentration, C_t , the concentration after t minutes, v , the volume of HCl in cubic centimeters, $e = 2.71$

² The writer is indebted to Dr. S. E. Hill for the design of this "push pull" vacuum tube amplifier.

Approximately half the volume of the solution (ca 500 cc) on one side was then removed and replaced by an equal amount of H_2O . After 6 to 7 minutes the mixing and diffusion had reached a steady state as indicated by the E.M.F. which, after that period, had approached a practically constant value. This E.M.F. was recorded and samples were taken for analyses. A new half part of the

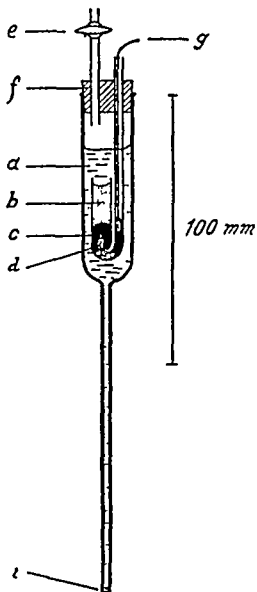


Fig 1 Compact type of calomel electrode *a*, saturated KCl, *b*, $Hg Cl_2$ in saturated KCl + 3 per cent agar agar, *c*, mercury, *d*, platinum wire, *e*, stop-cock, *f*, rubber stopper, *g*, lead to instrument (Cu wire), *i*, porous plug of sintered glass

solution was replaced by water, etc, the procedure being repeated until a dilution of about 1:16 had been measured.

The E.M.F. measurements are regarded accurate to ± 0.2 mv, the concentration figures (obtained by electrometric titration of Cl with $AgNO_3$) have a mean error of less than 1 per cent.

TABLES I-V

Apparent Cation Transference Number in Cellophane

1	2	3	4	5	6	7	8	9
Conc C_2	γ_2	Activity $C_2 \times \gamma_2$	E_{AgCl}	$(\epsilon_1 - \epsilon_2)^*$	π_{AgCl} (= 4-5)	E_{calom} (= π_{calom})	Difference 6-7	t^+ (AgCl)
mm/l			mv			mv		
I HCl (Temp 23.0°)								
C_1 98.4	0.80 ₁	78.7	0	—	—	0	—	—
50.8	0.83 ₆	42.4	+27.1	+15.7	+11.4	+12.0	-0.6	0.865
26.5	0.86 ₈	23.0	53.5	31.3	22.2	23.7	-1.5	0.856
14.3	0.89 ₆	12.8	80.2	46.2	34.0	34.3	-0.3	0.867
8.3	0.91 ₆	7.6	102.1	59.5	42.6	43.8	-1.2	0.859
II LiCl (Temp 24.2°)								
C_1 96.7	0.78 ₀	75.4	0	—	—	0	—	—
50.6	0.82 ₀	41.5	+12.1	+15.2	-3.1	-3.4	+0.3	0.398
25.7	0.85 ₈	22.1	25.6	31.4	-5.8	-5.1	-0.7	0.407
13.8	0.88 ₀	12.3	39.4	46.5	-7.1	-6.3	-0.8	0.424
7.43	0.91 ₄	6.81	52.2	61.7	-9.5	-7.5	-2.0	0.422
III NaCl (Temp 24.0°)								
C_1 96.0	0.78 ₀	74.8	0	—	—	0	—	—
49.9	0.82 ₁	41.0	+14.5	+15.4	-0.9	-0.6	-0.3	0.471
26.0	0.85 ₇	22.3	29.6	30.9	-1.3	-0.5	-0.8	0.479
13.8	0.88 ₈	12.3	45.4	46.3	-0.9	-0.7	-0.2	0.491
7.61	0.91 ₃	6.95	60.3	60.9	-0.6	-0.5	-0.1	0.495
IV KCl (Temp 24.0°)								
C_1 100.0	0.76 ₂	76.2	0	—	—	0	—	—
51.2	0.80 ₈	41.4	+17.2	+15.6	+1.6	+1.5	+0.1	0.551
26.8	0.84 ₉	22.8	35.0	30.8	4.2	4.5	-0.3	0.569
14.7	0.88 ₁	12.9	52.0	45.4	6.6	8.0	-1.4	0.573
8.16	0.90 ₈	7.41	68.7	59.6	9.1	10.9	-1.8	0.575
V NH ₄ Cl (Temp 22.2°)								
C_1 98.3	0.76 ₂	74.7	0	—	—	0	—	—
51.4	0.80 ₈	41.6	+17.1	+14.9	+2.2	+1.8	+0.4	0.574
26.6	0.84 ₃	22.5	35.0	30.5	4.5	4.7	-0.2	0.574
13.9	0.88 ₄	12.3	52.6	45.7	6.9	7.1	-0.2	0.574
7.67	0.91 ₁	7.0	69.3	60.2	9.1	10.8	-1.7	0.574
4.75	0.92 ₈	4.41	83.5	71.9	11.6	13.1	-1.5	0.581

$$* = 0.1983 T \log \frac{C_1 \gamma_1}{C_2 \gamma_2}$$

RESULTS

The figures obtained for the cellophane transference numbers of the cations in different chloride solutions over the concentration range 0.1 to about 0.007 *M* are recorded in the last column of Tables I to V. Only the t^+ values as computed from Equation 4, using AgCl electrodes, are given, however, because the corresponding figures computed for the "calomel" case according to Equation 2a would probably be approximately the same. As is indicated in Column 8, the "membrane potentials" as measured with calomel electrodes (Column 7) are almost consistently slightly more positive than those computed from AgCl measurements (Column 6). The main source of this difference

TABLE VI

Summary of Determined Transference Numbers and Mobility Ratios in Cellophane and a Comparison with Corresponding Figures in Free Water

Substance	Temperature	t^+ (cellophane)	t^+ (H ₂ O)	u/v (cellophane)	u/v (H ₂ O)	u/v u/v
HCl	23.0	0.863 ± 0.005	0.828	6.24 ± 0.25	4.81	1.30 ± 0.05
NH ₄ Cl	22.2	0.575 ± 0.002	0.491	1.35 ± 0.01	0.965	1.40 ± 0.01
KCl	24.0	0.567 ± 0.008	0.490	1.31 ± 0.04	0.961	1.36 ± 0.05
NaCl	24.0	0.484 ± 0.009	0.388	$0.94_0 \pm 0.03_4$	0.634	1.48 ± 0.05
LiCl	24.2	0.413 ± 0.010	0.323	$0.70_4 \pm 0.03_6$	0.477	1.47 ± 0.07

* Calculated as the mean of accurate figures given for 0.1 *N* and 0.01 *N* solutions by Longworth (12).

seems to be the fact that the stirring affects the "calomel potentials" far more than the "AgCl potentials". When the stirring was stopped the former became around 2 mv, the latter only about 0.2 mv more negative. Correction of E_{calom} for the "stirring effect" with ca. -0.8 mv would improve the agreement.

The (mean) activity figures employed in the tables are interpolated from data given by Harned (10) and Scatchard (11).

The t^+ values at the different concentration differences agree fairly well with each other and are constant within the limits of experimental error. In spite of the weak trend that can be observed and the fact that t^+ should theoretically vary with the concentration, the t^+ figures are averaged and summarized in Table VI. In the same table the

ratio "apparent cation mobility / apparent anion mobility" is given (calculated from Equation 3). The \pm terms indicate the average errors. For comparison corresponding figures for "free" water solutions are tabulated.

Table VI shows that the apparent *cation transference number in cellophane is higher* for all electrolytes here tested than it is in free water. Hence *the (apparent) mobility ratio between cation and anion is also increased in the cellophane to the same extent* (about 40 per cent for all cases).

DISCUSSION

The ionic transference numbers and relative ionic mobilities within a membrane determined and calculated as described above may have no actual physical meaning for many reasons, some of which have already been stressed by Michaelis *et al.* (2a) for the case of "dried collodion membrane." It seems desirable to emphasize certain of the points involved.

A glance at Equation 4³ shows that the transference number is determined from one *measured* quantity, the total $\Sigma \Delta F$ of the concentration chain, and from one *computed* quantity including the product, concentration \times activity coefficient = activity.

(A) In the first place, in our Equation 4, we assume that there are only the three potential "jumps," ϵ_1 , ϵ_2 , and π (*cf* Equation 1 and p 918), which make up the total $\Sigma \Delta F$. This assumption is somewhat doubtful when using a membrane like cellophane. Here the diffusion of the ionic constituents takes place in the presence of the electrically "polar" cellulose, one may consider the cellulose as a system of very narrow capillaries or as another "phase."

It may be possible that two extra potential jumps can exist, forming a "boundary" potential at each surface of the membrane. This possibility is not considered in the derivation of Equation 4.

(B) Furthermore, we have to expect that either the capillary dimensions, or the change of phase, as the case may be, can influence the thermodynamic activity of both the solvent and the solutes in the membrane. It is well known that the vapor pressure is different in the

³Michaelis *et al.* (2b) used with their procedure an equation corresponding to Equation 2a.

capillaries, etc., and the phase change may introduce changes in the dielectric constant, etc. Accordingly the procedure used, *i.e.* employing the activity as calculated for the bulk of the solution surrounding the membrane, may not be justified.

(C) As to the electrical effects exhibited by the membrane the following may be said

Besides the possibility already mentioned of the appearance of two boundary potentials (recently discussed by Wilbrandt (13)) the "charge" of the membrane may have influence upon the ionic migration. According to Michaelis' earlier ideas the charge was due to "adsorption" of ions. This view has been modified by Wilbrandt who attributes the charge to the effects of presence of polar groups in the membrane substance. Both authors seem inclined to think that the "mobility" of the ions can be greatly changed within a membrane. Their interpretation of the term mobility is not clearly defined, but evidently they mean "apparent rate of penetration." We will, however, use mobility in the sense as being an inverse function of the friction, as is the significance of the μ and ν in the diffusion equations, for instance Equation 2.

The ideas of Michaelis *et al.* regarding the repulsive or attractive action of the membrane charge upon ionic transport are of a purely qualitative nature. It seems, however, that a more quantitative and unitary treatment of the whole subject might be arrived at, if we regard the penetration of ions across a membrane as being a case of diffusion in an electrolyte *mixture*. Hitherto, only the concepts valid for *single* electrolyte diffusion have been applied, indicated, for instance, by the use of the simple Nernst formula, Equation 2. This can hardly be fully justified just because of the fact that the membrane may exert electrical forces. The membrane effect can, as a first approximation, be expressed as that of an *added* electrolyte affecting the diffusion of *other* electrolytes (those under investigation). For this more complicated case, Equation 2 is invalid. The treatment of the problem ought instead to be the same as that given long ago for the mutual effects in cases of diffusion in electrolyte mixtures by Planck (14), Arrhenius (15), Henderson (16), and many others. For these theories it is immaterial what sources the charges of the solutes have. Accordingly the effects predictable from these concepts would be of

a perfectly general nature and not limited to simple cases of free ionic diffusion

A fuller presentation of membrane permeability analyzed from the viewpoint of "forced" diffusion of mixtures has to be postponed to a separate communication. It can be said, however, that the present preliminary calculations, taking into account an electrolyte behavior of the membrane, show the possibility that *the total membrane potential may be increased or decreased with the real ionic mobilities of the diffusing substance remaining the same as in free water*⁴. From the discussion above it can be concluded that the computation of the "transference number" from the total EMF, as has been outlined on pp 918 and 919, may be misleading because too simple a formula has been applied in the derivations, *i e*, Equation 2

It is felt, however, that the transference number data obtained here can be used at least for approximate calculations of the relative ionic mobilities within cellophane, provided they are applied to cases having concentration figures of the same magnitude as those employed in the present experiments, *i e*, 0.1 to 0.01 normal

SUMMARY

The "apparent" cation transference number within cellophane is determined for HCl, KCl, NH₄Cl, NaCl, and LiCl

The method consists in measuring the EMF in a concentration chain employing Ag/AgCl electrodes or calomel electrodes and calculating from formulas derived for cases of simple, unconstrained diffusion

The transference numbers and the cation mobilities relative to the chloride ion were found to be higher in the cellophane (relative cation mobilities increased about 40 per cent)

The effect of the membrane is discussed. It is emphasized that with the introduction of a membrane as a liquid junction new factors are introduced, which are not considered in the formulas ordinarily used. Such factors may be activity changes due to dimensional or other reasons and particularly electrical effects exhibited by the membrane

⁴A simplified theory is given by the writer (18)

upon the ionic diffusion. Accordingly the transference number, as determined, may lack well defined physical significance.

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EXPERIMENTAL PROCEDURE

The electrical measurements were made by the method previously described (4), which consisted of using a Wheatstone bridge to indicate the electrical equivalence of the suspension with a salt solution of the same resistance in parallel to a variable air condenser. The resistance of the salt solution is then the resistance of the suspension, and the capacity of the suspension is the capacity of the condenser plus the static capacity of the salt solution. Care was taken to eliminate electrode polarization. Measurements were made at a number of different frequencies, but the computations were all made with a frequency of 16 kilocycles per second, the other measurements merely being taken to make sure that the variation of capacity with frequency was the same as for normal blood.

Having measured the capacity per cm^2 of suspension (C), specific resistance (R), and specific resistance of suspending fluid (R_1), it is possible by means of a formula developed by Cole (5) to compute the capacity for a 100 per cent volume concentration, namely

$$C_{100 \text{ per cent}} = \frac{3C}{\left(1 - \frac{R_1}{R}\right)\left(2 + \frac{R_1}{R}\right)}$$

Fricke (1) has shown that it is then possible to compute the capacity per cm^2 of membrane (C_o) by means of the formula

$$C_o = \frac{C_{100 \text{ per cent}}}{\alpha q}$$

where α is a factor¹ dependent on the shape of the cell, and $2q$ is the length of the major axis of the cell. The thickness is given by the formula

$$t = \frac{D}{4\pi C_o} \cdot \frac{10}{9} \cdot 10^{-6}$$

where D is the dielectric constant. The thickness will be in centimeters if the value of C_o is given in μf .

In all cases the initial volume of the individual cells is taken from measurements by Ponder (6). For the swollen cells, it is a simple matter to compute the final cell volume, knowing the initial volume concentration, the exact amount of fluid added, and the final volume concentration. All volume concentrations were determined by the electrical method (7).

¹ For normal rabbit red cells $\alpha = 1.28$, and for spherical cells $\alpha = 1.50$

EXPERIMENTAL RESULTS

The results for the experiments performed to determine the effect on the membrane capacity of a change in shape showed in every case an increase in capacity upon being made spherical. For example, in one experiment normal rabbit corpuscles, washed three times in 1 per cent NaCl, were found to have a membrane capacity of $0.91 \mu\text{f}/\text{cm}^2$, and volume concentration of 34.4 per cent. To this suspension was added 0.068 cc of M/1000 rose bengal per cubic centimeter of suspension. All cells appeared spherical under the microscope. The membrane capacity was measured to be $1.09 \mu\text{f}/\text{cm}^2$. The average for the normal cells is $0.96 \mu\text{f}/\text{cm}^2 \pm 10$ per cent² and for spherical cells $1.07 \mu\text{f}/\text{cm}^2 \pm 10$ per cent.

The experiments performed to determine the change on swelling showed in each case a slight decrease in capacity as the cells were swollen. In one experiment the cells were made spherical as described above and found to have a capacity of $0.99 \mu\text{f}/\text{cm}^2$, and a volume concentration of 38.3 per cent. To this suspension was added 0.60 cc of 0.30 per cent NaCl per cubic centimeter of suspension and the volume concentration was now found to be 30.5 per cent and the membrane capacity $0.87 \mu\text{f}/\text{cm}^2$. From the volume concentrations it will be seen that there has been a swelling of 27 per cent which represents an increase in area of 17 per cent.

DISCUSSION

Thus the values of C_0 for spherical cells are somewhat larger than those for normal cells. It is not known whether this difference is real or not. In the first place the formulae assume the normal red cell to be a perfect spheroid, and since it is not, a small error is introduced. In the second place, if an appreciable number of cells in an experiment were not completely spherical, which seems entirely possible, then the value would appear higher even though there were no change. It can be definitely stated, however, that there is no great change, and if there is a slight change, it is in the direction which would indicate that the

² The value of $C = 1.10 \mu\text{f}/\text{cm}^2$ for normal corpuscles previously published (4), was given on the assumption that there was no real capacity difference between normal and spherical cells, and that the value obtained for the spherical shape was probably more nearly correct.

membrane is thinner. When lecithin or rose bengal is added to a suspension of red cells, the added substance is adsorbed by the membrane, enough so that it has been estimated that the membrane is covered by at least a monolayer of the substance. It might easily be expected that this would show up in the capacity measurements as a thickening of the membrane. Since this is not the case, it must be concluded that either there is a change in dielectric constant to compensate for the thickness change, or the membrane is so thick that the addition of a monolayer does not change its thickness appreciably, or that the added substance has such a high conductance or high dielectric constant that the electrical properties of the membrane are not appreciably affected by its presence.

In the swelling experiments it might be expected that in view of the swelling and stretching of the membrane the capacity of the membrane should be larger. The measurements, however, indicate a slight decrease. Since the corpuscles are spherical both before and after swelling, the computations are more exact, and it is believed that the decrease in capacity is real, but just outside the experimental error. These results are in complete accord with the measurements of Cole (8) who found that the membrane capacity for *Hippovoe* eggs decreases on stretching by about the same amount as found here for red cells. However, in his experiments the swelling could be carried much further without injury to the cell, so that the decrease seemed more certain. This would seem to indicate that as the cell swells the membrane does not stretch but new material comes from the interior of the cell to make a new portion of the membrane. This is in accord with the theory of Ponder (9).

The author is indebted to Drs. Eric Ponder and Hugo Fricke of this laboratory for assistance and cooperation in this work.

SUMMARY

Measurements of the static capacity per cm^2 of membrane for the red corpuscle as changed when the cells are made spherical by the addition of lecithin or rose bengal, show a slight increase of capacity, indicating a thinning of the membrane, although the change is not large enough to make it certain that it is real. Furthermore, the

membrane capacity shows a slight decrease when spherical cells are swollen in hypotonic saline, indicating a thickening of the membrane, although the change is hardly outside the experimental error. The fact that there is no increase in capacity lends support to the theory that as the cell swells the membrane does not stretch but new material comes from the interior of the cell to make a new portion of the membrane.

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ADAPTATION OF CUTANEOUS TACTILE RECEPTORS

IV ELECTROLYTE CONTENT OF FROG SKIN

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Hoagland (1934, 1935 *a, b*) has presented evidence indicating that the failure of response of the tactile axon endings in frog's skin to mechanical stimulation of the skin may be due to reduction of the excitability of the endings by the release of potassium from surrounding epithelial cells when they are pressed upon. To test this hypothesis it was desirable to obtain information concerning the electrolyte content of the skin areas used in these experiments. Up to the time of the present investigation there seemed to be no available data describing the electrolyte content of the skin of the back and sides only of *Rana pipiens*.

A sample of freshly removed skin (approximately 0.2 gm.) from the back or sides was blotted lightly on smooth filter paper, placed in a porcelain crucible, and weighed. The sample was dried to a constant weight in a desiccator and reduced to a white ash in a muffle furnace. Except in the case of the chloride analyses, the ash was taken up in 50 cc. of 0.1N HCl. For chloride analysis the ash was dissolved in hot water to which was added a few drops of concentrated H_2SO_4 . The following colorimetric procedures were employed with but slight modifications as conditions required: *potassium*—Jacobs and Hoffman (1931), *sodium*—Salt (1932), *calcium*—Roe and Kahn (1929), *phosphate* (inorganic)—Bell and Doisey (1920), *sulfate* (inorganic)—Wakefield (1929), *magnesium*—Yoshimatsu (1929-30) *chloride*—McClendon (1934).

Table I shows the results obtained by the analyses. The values are typical for both the back and the sides, since no significant difference was observed between them. Only a few analyses were performed on some of the constituents since the purpose of this investigation was merely to obtain relative concentrations, and especially to see if potassium were concentrated in the skin in such a way as to be in harmony with the proposed adaptation hypothesis.

From the table it is evident that there is considerable variation in the electrolyte content from animal to animal, especially of Ca and P. The averages of these values give relationships which are useful for our purpose.

The ratio of K in the skin to the K in frog plasma is of the same order of magnitude as that found by Fenn, Cobb, Hegnauer, and Marsh (1934) between frog nerve and plasma, $\frac{\text{Skin K}}{\text{Plasma K}} = \text{about } 14$, and $\frac{\text{Nerve K}}{\text{Plasma K}} = \text{about } 19$. If, among other things, the excitability

TABLE I

The following values are expressed in milligrams of electrolyte per hundred grams of wet tissue. Experimental errors as follow: K ± 2 per cent, Na ± 2 per cent, Ca ± 7 per cent, Mg ± 3 per cent, Cl ± 1 per cent, S ± 5 per cent, P ± 2 per cent.

K	Na	Ca	Mg	Cl	S	P
<i>mg per cent</i>	<i>mg per cent</i>	<i>mg per cent</i>	<i>mg per cent</i>	<i>mg per cent</i>	<i>mg per cent</i>	<i>mg per cent</i>
136.0	86.9	340.0	3.8	182.2	36.3	341.9
124.0	94.3	310.5	5.0	178.1	29.9	319.0
128.5		205.0		187.6		271.2
131.6		354.9		139.5		318.7
141.8		328.4				438.1
		258.6				410.3
		419.2				310.5
		440.9				307.5
		262.5				321.1
Average 132.4	90.6	302.2	4.4	171.9	33.1	337.6

of the axon endings is dependent on the ratio of potassium inside the axon to that outside in the plasma (K_i/K_o), then the release of K from the epithelial cells surrounding the endings would decrease their excitability by reducing the ratio K_i/K_o . The amount of K present in the skin is consistent with such an hypothesis.

The amount of Na present in the skin indicates that probably an insignificant amount of it would be released from the skin on mechanical stimulation, because it would be opposed by an appreciable concentration gradient. The Na concentration of the plasma is about 2.6 times as great as is that in the skin. The Ca, although

present in considerable amounts, is probably present in the skin in an indiffusible form ($\text{Ca}_3(\text{PO}_4)_2$). As will be shown in a subsequent paper, Ca is not expressed from the skin under pressures used in Hoagland's adaptation experiments. Mg is present in very small amounts, and, if anything, might be expected to increase the excitability (Schaefer, 1934). The anion Cl and the S present would not be expected to have any effect on the excitability of the axon endings.

SUMMARY

The potassium content of the skin of *Rana pipiens* is found to be 132 mg per cent. This is roughly of the order of magnitude of the content of potassium in nerve.

Analyses were also made of the skin for Na, Ca, Mg, Cl, S, and P.

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ADAPTATION OF CUTANEOUS TACTILE RECEPTORS

V THE RELEASE OF POTASSIUM FROM FROG SKIN BY MECHANICAL STIMULATION*

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The hypothesis that the liberation of potassium from the epithelial cells of frog's skin is responsible for the adaptation of the tactile endings (Hoagland, 1934, 1935 *a, b*) raises the question whether potassium is actually liberated from the skin on stimulation. The following experiments were devised to test this.

A piece of skin 1 x 2 cm. was cut from the back of a frog and placed in Ringer's solution for 5 minutes. The Ringer's was continually agitated in the region of the skin so as to insure complete washing away of electrolytes released from the cut epithelial cells. The skin was then mounted inner side down, on a glass microscope slide by means of two rubber bands and again washed with Ringer's to eliminate electrolytes that might have been squeezed out of the skin as a result of the manipulation involved in mounting it. Several pieces of fine glass rod were cemented onto the microscope slide lengthwise, giving a corrugated effect, to allow washing the under side of the skin with a stream of Ringer's delivered by a medicine dropper the tip of which was pulled out to a small diameter. The slide with the skin was placed vertically in a small glass container and the air jet used in the stimulating experiments previously described (Hoagland 1933) was directed on the skin. 10 cc. of Ringer's solution was placed in the vessel and the air jet was turned on, producing repetitive stimulation of a spot at a frequency of 140 per second with a nozzle pressure of 400 mm. Hg. During stimulation lasting for 3 minutes the 10 cc. of Ringer's was repeatedly streamed down the under side of the skin by means of the 2 cc. medicine dropper. Some twenty such washings were delivered during the 3 minutes that the stimulation lasted. The "wash" was then collected in a clean vial and tightly stoppered to be analyzed later.

Control experiments were carried out in the following manner. A sample of skin was washed and mounted on the slide in the same way as previously described.

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The skin was then given twenty 2 cc washes with 10 cc of Ringer's but without being stimulated by the air jet. The Ringer's from these control experiments was collected and then analyzed along with the other washes.

To prevent personal bias from entering the analyses, only one of the authors knew which of the samples was from a control or a "beaten" experiment. The vials were numbered and given to the other investigator (M A R) for analysis. Only after the analyses were completed did the analyst learn the nature of the contents of the vial. Occasionally samples of plain Ringer's solution were placed among the vials without the knowledge of the analyst.

The wash was analyzed only for potassium, calcium, phosphate, and chloride ions. The analytical procedures were those indicated in a previous paper (Rubin, 1936), with the exception of calcium. For calcium the method described by Cameron and White (1930) was used. Table I shows the results of the analyses. The term "beaten" in the table refers to samples of wash from skin stimulated by the interrupted air jet.

TABLE I

Ion	No beaten analyses	Excess in wash (average)	Extremes	No control analyses	Excess in wash (average)	Extremes
		mg per cent	mg per cent		mg per cent	mg per cent
K	11	0.67	0.33-1.16	13	0.06	0.03-0.11
Ca	6	None	None	5	None	None
PO ₄	4	None	None	4	None	None
Cl	4	None	None	4	None	None

The mean excess of 0.67 mg per cent of K is equivalent to an increase of K in the Ringer's of 9 per cent. Experimental error ± 2 per cent.

From Table I it is evident that, of the four ions investigated, potassium is the only one passing out of the skin on stimulation with the air jet. It is possible that some other ions may be "squeezed" from the epithelial cells and do reach the nerve endings in the region of stimulation, but they must be almost negligible in quantity as compared with potassium, since the latter passes out into the wash. No attempt was made to analyze the wash for other ions because the four ions investigated are the most abundant in the skin, and the other ions would not be expected to have as an appreciable inhibitory effect on the excitability of the tactile nerve endings as does potassium.

Potassium in frog's skin is extremely diffusible. This can be demonstrated by soaking a piece of skin in about ten times its weight of distilled water. In six such experiments (Table II) the amounts

($128 \pm$ mg per cent of skin) found in the water at the end of 2 hours are nearly equal to that found in the skin itself. The skin on the back and sides of *Rana pipiens* contains about 133 mg per cent potassium (Rubin, 1936).

That potassium is released from the epithelial cells of frog's skin when it is stimulated by the air jet may be regarded as established by these experiments. Calcium, phosphate, and chloride do not appear to be released. There still remain the possibilities that (1) other inorganic ions may be freed from the epithelial cells and exert some influence in very small concentrations, although it is improbable as Hoagland (1936) has demonstrated that only calcium has an effect on the tactile endings similar to that of potassium, but the effect is

TABLE II
Potassium Dialyzed from Frog's Skin by Distilled Water

Mg K/100 gm. wet skin	
	135.3
	126.7
	131.9
	120.5
	128.8
	124.8
Mean	128.0

essentially irreversible, while the potassium effect is readily reversible, or (2) some organic humor (acetylcholine (?), histamine (?)) may be involved to complicate the picture. Preliminary evidence has been obtained, to be presented in a subsequent paper, which indicates that something, probably of an organic nature, may be liberated from the frog skin when it is beaten by the air jet and to a lesser degree when it is washed without the beating. It is, however, quite possible that this substance may have very little, or nothing, to do with adaptation.¹

¹ Since this paper went to press one of us (M A R., unpublished) has precipitated potassium in frog skin by a modified form of Macallum's method. The potassium, in the form of orange yellow crystals of potassium sodium

SUMMARY

Potassium is released from the epithelial cells of frog's skin on stimulation by an interrupted air jet. This evidence is consistent with the hypothesis that potassium is involved in the adaptation of the tactile nerve endings in frog's skin.

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-

is concentrated in the epidermis, a little is found around the gland cells, and very little, if any, in the corium. The free, tactile endings, which adapt rapidly, terminate between the epithelial cells and are, therefore, in close proximity to cells most rich in potassium, the slowly adapting pressure receptor endings (Hogg, 1935) are found in the corium (Syrocki, unpublished).

ADAPTATION OF CUTANEOUS TACTILE RECEPTORS

VI INHIBITORY EFFECTS OF POTASSIUM AND CALCIUM*

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I

In previous papers in this journal evidence has been presented indicating that potassium released from cutaneous cells of frog's skin, when the skin is stimulated by pressure, produces a reversible failure of responsiveness of the free nerve endings serving as tactile receptors anastomosing among the cutaneous cells. Fig 1 shows schematically an antidromic single axon (*cf* Adrian, Cattell, and Hoagland, 1931). In brief, the evidence for the potassium hypothesis of adaptation is based principally on the following findings

The cutaneous cells are rich in potassium (Rubin, 1936). Isotonic KCl solutions applied to the underside of the skin containing a single axon ending greatly hastens the rate of failure of the ending to respond to an interrupted jet of air impinging on the outer side of the skin. Washing with Ringer's solution results in recovery from adaptation both when this is produced by the localized repetitive air jet stimulation and when the adaptation had been hastened by washing with a solution of isotonic KCl (Hoagland 1934).

Adaptation is not produced by electrically initiated antidromic impulses back fired over the axon branches and therefore is not the result of the activity of the axon branches *per se*. The time curves showing production of and recovery from adaptation are inconsistent with any known properties of peripheral nerve. In intermittent air jet stimulation of a region of skin several millimeters distant from a responsive single ending produces failure of response of the ending to a similar direct intermittent stimulus applied to the skin area containing the ending immediately afterward.

This indicates the passage of an inhibitory humor from the first region not

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supplied by the particular axon under investigation to the second stimulated region containing the axon ending (Hoagland, 1935 a) When freshly removed pieces of skin are mounted in front of the interrupted air jet and stimulated, and at the same time are washed with Ringer's solution, it is found that these washings are always richer in potassium than are similar washings made without the air jet stimulation Potassium is thus shown to come out of the stimulated and washed skin but does not do so when the skin is merely washed but not stimulated Calcium is not found to be washed out of the "beaten" skin (Hoagland and Rubin, 1936)

These experiments indicate that the failure of the endings is due to raising the concentration of potassium, K_o , outside of the axon branches, which thus reduces their excitability by lowering the ratio of potassium inside the nerve to that outside, (K_i/K_o) These ideas have been extended to account for adaptation in other mechano-receptors (*cf* Hoagland, 1935 b)

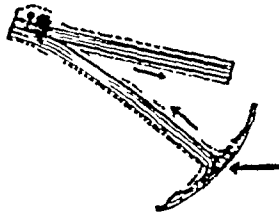


FIG 1 Arrangement of nerve fibers giving the antidromic discharge Leads are placed on the dorsal cutaneous nerve cut at its entrance to the skin The antidromic touch spot is usually located ventrally The bifurcating fiber branches in the dorsal root ganglion

II

In the present experiments an attempt has been made to determine the effects of applied Ringer solutions rich in K or Ca on the responsiveness of single axon endings The amount of K in frog's nerve has been shown to be of the order of $188 \pm \text{mg per cent}$ (Fenn, Cobb, Hegnauer, and Marsh, 1934) This is about fifteen to twenty times greater than the K content of frog plasma Accordingly a solution was made up consisting of Ringer's solution plus fifteen times the normal K content If this solution could be applied directly to the endings it should reduce the K_i/K_o ratio substantially, and hence inhibit the excitability. Even if the cutaneous cells surrounding the axon endings prevented the K from reaching them at this concentration some decline in responsiveness of the endings might be expected Conceivably, pressure on the skin could release enough potassium from

cells in intimate proximity to the axon branches (Hoagland and Rubin, 1936) to reduce the concentration ratio, K_i/K_o , to unity

To compare the effects of Ca on the responsiveness of the axon branches, solutions of Ringer plus fifteen times the normal Ca content were also used. It seemed desirable to test the effect of excess of Ca on adaptation directly, despite the finding that Ca is not washed out of the skin when it is "beaten" with the air jet (Hoagland and Rubin, 1936). We were not interested in sodium effects since excessive amounts of Na in Ringer have not been found to produce appreciable effects on adaptation. Frog plasma contains 239 mg per cent of Na (Fenn, Cobb, Hegnauer, and Marsh, 1934) and frog skin 91 mg per cent of Na (Rubin, 1936). Therefore, we should not expect that the high local plasma content of Na surrounding a free-ending axon would be appreciably augmented by excretion of Na from epithelial cells under deforming pressure.¹

The antidromic preparation was similar to that used in previous experiments (Hoagland, 1934). The air jet was arranged to impinge upon a single "antidromic spot" in the skin (*cf.* Fig. 1) of a completely pithed frog. Short slits were cut in the skin above and below the spot, about a centimeter apart, and a bulb pipet was used for passing the solutions across the underside of the skin. The single axon impulses set up in response to the interrupted air jet stimulus were recorded optically and audibly by means of a Matthews oscillograph and loud speaker.

The procedure consisted of passing normal Ringer's solution across the underside of the skin and then stimulating with the air jet at a pressure of approximately 400 mm of Hg with the nozzle clamped rigidly 5 to 10 mm above the spot. The frequency of stimulation, 140 per second, was regulated by a notched disc rotating between nozzle and skin. Owing to the large number of adaptations measured it was not expedient to photograph the impulses. Instead, the time to failure of impulses was taken with a stop watch by listening to the loud speaker and checking the audible recording by observing the impulses on a standing wave screen. Occasionally photographic verifications were made of the reliability of this method.

¹ Talaat (1933) studied the effect of ions on the excitability of the axon endings in frog's skin. He found adaptation to be greatly retarded if Ca were removed from the Ringer's solution. Citrate and oxalate in Ringer's bathing the skin prolong the discharge by removing Ca. These experiments have little bearing on the present studies since they involved removing ions from the bathing fluids and testing the effects on axon excitabilities. In the present experiments bathing solutions were made with ionic properties similar to those called for in conformity with the hypothesis that adaptation may be due to the release of ions from cutaneous cells to plasma under pressure. The solutions therefore, contained certain ions in excess of those in Ringer's solution. In Talaat's experiments the removal of Ca might be expected to alter profoundly the excitabilities of nerve membranes quite independently of the K_i/K_o effect.

When adaptation had occurred the air was turned off and exactly 75 seconds were allowed for recovery (Hoagland, 1934). During this time the underside of the skin containing the spot was again washed with Ringer's solution. In each case about 8 cc. of solution were used at each washing. At the conclusion of the rest period the stimulus was again applied and the adaptation was measured again. In this way it was possible, by obtaining successive readings, to determine the time to adaptation for a single axon in a region of skin bathed in Ringer's solution.

Using this procedure the solutions containing excess K or excess Ca were applied from time to time and the effects of these solutions on the time to adaptation were measured. Regardless of which of the three solutions was used, 75 second rest intervals were always allowed to elapse between successive stimulations.

Figs. 2 and 3 show the results of typical experiments. The total time intervening from the beginning of the experiment to a given adaptation determination is plotted as abscissa against the time to adaptation of each successive stimulation as ordinate. The figures are labeled to show which of the three solutions—Ringer's, Ringer's with $(15 \times K)$, or Ringer's with $(15 \times Ca)$ —were used to wash the skin in the 75 second rest interval prior to the succeeding adaptation determination.

Only about 20 per cent of some hundred single ending preparations isolated in these experiments showed sufficient durability for our purposes. It is necessary for an ending and its nerve to survive for at least an hour and to give, when the skin is bathed in Ringer's solution, approximately a constant adaptation time. In all of the experiments it was necessary to place the dorsal cutaneous nerve used for recording in a moist chamber. This was done by packing Ringer-soaked cotton around the nerve as it emerged from the frog's back and passed to a glass tube containing the lead electrodes. The reasons for failure of the endings are unknown. Usually the failure is abrupt, all responsiveness suddenly disappearing. In some cases the nerve fiber in the region of the electrodes clearly failed since the impulses gradually decreased in amplitude, although the ending gave normal responses to the intermittent stimulus. Some of the tested endings showed considerably more variation in their normal adaptation times than those shown in the published figures. Qualitatively, however, they bore out the results depicted in the figures.

The following generalizations may be made from Figs 2 and 3

1 Bathing the skin in solutions of Ringer plus fifteen times the normal K content greatly hastens subsequent adaptation The depression of excitability is usually immediate Subsequent applica

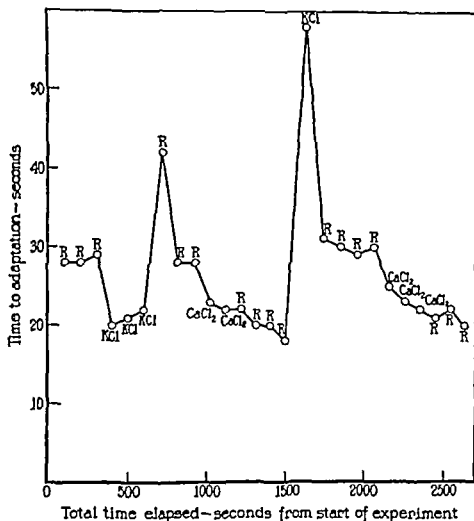


FIG 2 Plot showing the effect on the excitability of a single axon ending to solutions of Ringer, of Ringer plus fifteen times the normal K content, and of Ringer plus fifteen times the normal Ca content The points on the figure are accordingly labeled R, KCl, and CaCl₂ to indicate which solution was used to bathe the skin prior to that particular adaptation determination

For further discussion see text

tions of the K rich solution do not cause appreciable decrements in the time of adaptation

2 Washing with Ringer's solution not only removes the inexcitability of the ending produced by K but usually produces a compensatory rebound in the ending's responsiveness, the ending reacts

longer time to the same stimulus after K depression and recovery produced by washing with Ringer's than it normally does

3 Application of solutions containing fifteen times the amount of Ca in Ringer's solution also produces a depression of responsiveness corresponding in magnitude roughly to the K depression. The Ca depression, however, is usually not produced so rapidly, several suc-

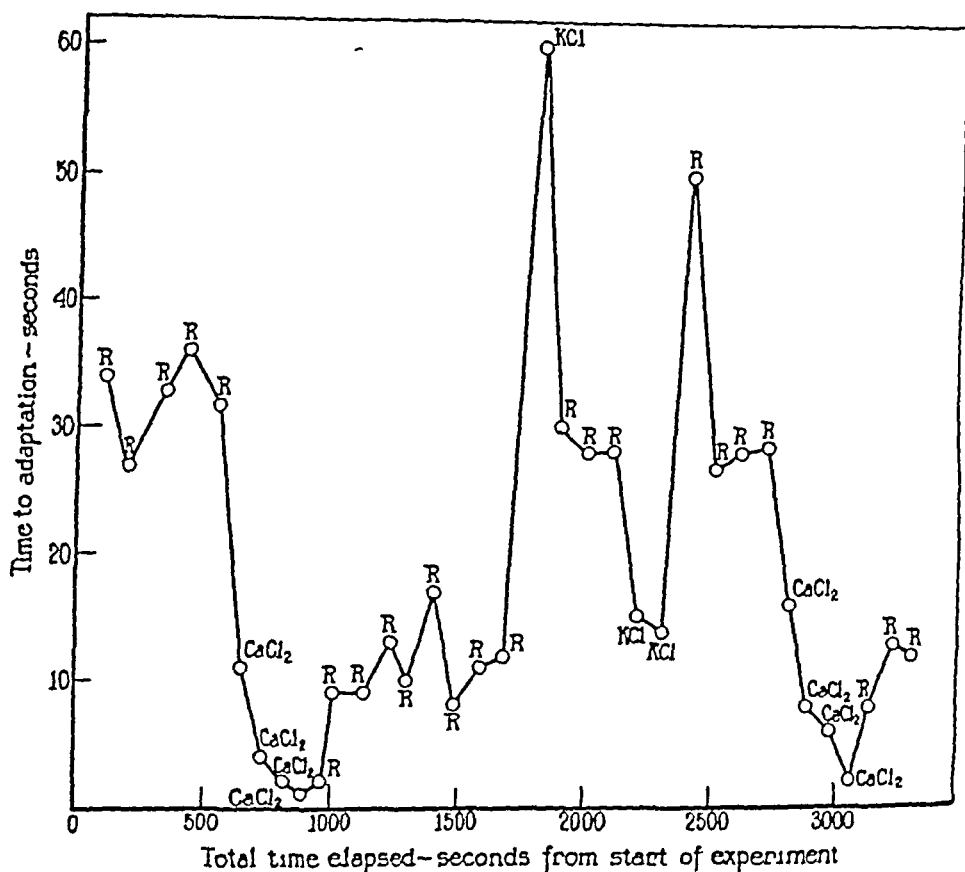


FIG 3 See legend to Fig 2

cessive applications showing progressive increments in the decline of the response

4 Washing with Ringer's solution produces either very little (Fig 3) or no recovery (Fig 2) of endings made less responsive with solutions containing an excess of Ca. This irreversibility in Ringer's of Ca depression is sharply in contrast with the depression produced by

K which is reversible in Ringer's. It suggests that different mechanisms are involved in the depressive actions of the cations. Talaat (1933), it should be recalled, found that absence of Ca greatly prolongs the time to adaptation.

5 When solutions of $15 \times K$ are applied after depression produced by solutions containing $15 \times Ca$, an immediate recovery occurs from the Ca depression. In this case there is also a compensatory rebound, the response lasting considerably longer than normal. This antagonistic recovery is especially interesting owing to the inhibitory action of solutions of $15 \times K$ when applied to the endings without being preceded by the Ca solution.

6 When depression of responsiveness of an ending is produced by a solution of Ringer's plus $15 \times K$, the application of a solution of Ringer's plus $15 \times Ca$ does not produce recovery as described in the foregoing paragraph. This effect is not shown in the published figures but was clearly seen in several of the preparations yielding the above typical results.

III

The inhibitory K effect illustrated by the foregoing experiments is quite consistent with what one would expect in connection with the potassium mechanism of adaptation reviewed at the beginning of this paper. The fact that Ca also inhibits the responsiveness of the endings suggests the possibility that this substance may normally be involved in adaptation since cutaneous cells not only store K (132 mg per cent) but also Ca (302 mg per cent) (Rubin, 1936), and conceivably Ca might be released when pressure is applied to the skin and thus inhibit the endings.

There are, however, several reasons which make it seem highly improbable that Ca is normally involved in adaptation. Hoagland and Rubin (1936) showed that Ca is not washed out of the skin in detectable amounts when the frog's skin is beaten with the air jet. They found, however, appreciable quantities of K released under these conditions. The K inhibitory effect occurs rapidly and apparently completely after one application of the K rich solution, while the Ca inhibition is usually slower in developing, taking distinctly longer to produce adaptation than does a comparable amount of K. Probably

the most interesting fact is that the Ca inhibition is essentially irreversible in Ringer's solution. This irreversibility is sharply in contrast with the abrupt reversibility of both the normal recovery from adaptation and recovery from adaptation hastened by the addition of K rich solutions. In view of these facts it would seem justifiable to conclude that Ca is not normally involved in the mechanism of adaptation of the free ending receptors. At present there seems to be no satisfactory explanation of the rebound of recovery above the normal level shown in the figures.

SUMMARY

1 Both solutions of Ringer plus fifteen times the normal K content, and solutions of Ringer plus fifteen times the normal Ca content markedly hasten the adaptation of single freely branching axon endings in frog's skin to repetitive air puff stimuli.

2 The K effect is produced more rapidly than is that of Ca. The K effect is reversible by washing with Ringer's solution, while the Ca effect is not. The Ca inhibition can, however, be reversed and recovery effected by washing with K rich solutions.

3 Evidence is discussed which indicates that Ca probably plays no rôle in normal adaptation, and the experiments are interpreted as substantiating the hypothesis of adaptation due to K.

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ON THE QUANTITY OF ELECTRICITY AND THE ENERGY IN ELECTRICAL STIMULATION

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The quantity of electricity and the energy have been assigned considerable significance in the problem of electrical excitation on account of the circumstance that these quantities appeared in or were easily derivable from two noteworthy early attempts to correlate the strength duration relations, namely, the laws of Weiss (1901) and Hoorweg (1892)¹ It does not appear that the significance of these laws has been discussed adequately with respect to the dynamics of the excitatory process There arises the question, therefore, as to whether the origin of these ideas about quantity and energy has a logical basis, and if it has not, there is another question as to whether any special significance can be assigned to the quantity and the energy from a point of view which is in conformance with more recent ideas about the nature of the excitatory process It is the present purpose to consider these questions

Weiss's law is easily derived as follows let

$$\frac{dp}{dt} = KV - k \quad (1)$$

where p is the state of excitation, V is the applied voltage, and K and k are constants If it is assumed further that a constant amount, h , of the local excitatory state is required for excitation, Equation (1) gives on integration,

$$Vt = \frac{h}{K} + \frac{k}{K}t \quad (2)$$

which is Weiss's law

¹ A quantity law by Dubois of Bern appeared earlier than these but it was soon found to be inadequate (see Lapicque 1926 Hoorweg, 1892)

Weiss's law, therefore, is the logical consequence of the assumptions, that the rate of growth of the excitatory state varies as the strength of the stimulus, that there is a simultaneous spontaneous subsidence of the excitatory state at a constant rate, k , and that equal amounts of the excitatory state are adequate for all threshold stimuli. The second, at least, of these assumptions is probably invalid for it implies that following an inadequate stimulus ($KV = 0$ in (1)), the local excitatory state subsides at a constant rate, while the data of latent addition are interpreted as indicating the existence of an exponential rate, *e.g.* Chauchards (1925), Lapicque (1925). It must be admitted that the direct information on latent addition is far from satisfactory but there is very good indirect evidence of the exponential subsidence which will be discussed later. It is conceded in addition that equation (2) represents the data rather poorly (Lapicque, 1926). It is therefore very likely that the basis of Weiss's law is unsatisfactory and that any derivations from the law will be equally unsatisfactory.

In particular with regard to the minimal energy, which is easily shown from (2) to occur when $t = \frac{h}{k}$ and $V = \frac{2k}{K} = 2R$, R , the rheobase, being the minimal voltage for times indefinitely long, it cannot be expected either that the experimental minimal energy will occur at exactly $V = 2R$, or that the duration for which it occurs can be related through Weiss's law to any parameter of the process of excitation.

It has been shown already by Strohl (1932) that Weiss's and Hoorweg's laws are inconsistent. His reasoning was on the basis of the quantities of electricity, but it can be shown equally well by the present method. For, if Weiss's and Hoorweg's laws have a common basis, the latter should be derivable, using the same assumptions as previously, from the equation,

$$\frac{dp}{dt} = KV e^{-\frac{t}{cr}} - k \quad (3)$$

where V_0 is the initial voltage of the stimulating condenser and l and r , are the capacity and resistance, respectively, of the circuit. The solution with $p = 0$ at $t = 0$ is

$$p = crKV_0 \left(1 - e^{-\frac{t}{cr}} \right) - kt \quad (4)$$

This function, p , has a maximum value at a time $t_0 = cr \log \frac{KV_0}{k}$. If $p = h$ at this time, t_0 , the utilization time of the discharge, it will be just adequate. These conditions in (4) give

$$h = cr KV_0 \left(1 - \frac{k}{KV_0}\right) - crk \log \frac{KV_0}{k} \quad (5)$$

but this is not Hoorweg's law. Therefore the bases of these laws are not the same.

It was pointed out by Strohl (1932) that putting the utilization time, $t_0 = \alpha cr$, α , a constant, gives the form of Hoorweg's law. In this case this is equivalent to putting $\log \frac{KV}{k} = \alpha$, which gives from (5)

$$V_0 = \frac{h}{Kcr} + \frac{k}{K}(1 + \alpha) \quad (6)$$

which is Hoorweg's law. The assumption above concerning the utilization is, however, entirely illogical with respect to the present argument as it implies that the excitatory state is more adequate at some value other than its maximal. Thus if it appeared desirable to extend Weiss's law it should be done by applying (5) to condenser data. The application of Hoorweg's law, equation (6), is quite irrelevant.

With regard to the question as to whether Hoorweg's equation may be derived from some basis other than equation (3) by a more logical assumption than that the utilization time is proportional to the cr of the circuit, independently of the voltage, it will be observed that any basis in which the growth of the excitatory process depends on the current or voltage of the discharging condenser involves a term

$e^{-\frac{t_0}{cr}}$. Since the integral of this expression will also contain $e^{-\frac{t_2}{cr}}$, and since Hoorweg's equation does not, it follows that some assumption has to be used which will get rid of it. The necessary assumption is $t_0 = cr \times \text{constant}$. It therefore seems unlikely that Hoorweg's law can be given a better interpretation than has been given. This conclusion does not apply, of course, to empirical integral expressions equivalent to Hoorweg's law in which various interpretations can be

given to the parameters without regard to the underlying dynamics. Such an expression is given, for example, by Ebbecke (1927)

It seems, therefore, that lacking a consistent common basis, the quantity laws and the energy relations derived from them are likely to be very misleading. Since they show, however, approximate relations involving the quantity and the energy, it will be of interest to examine a more exact representation of the data in order to determine whether or not these quantities will appear in a simple way. The writer's representation (1932) is chosen because it is the only one which has been shown to be consistent for more than one type of stimulus on the same preparation (1935 *a*, *b*)

The basis of this representation is the equation,

$$\frac{dp}{dt} = KV - kp \quad (7)$$

in which the symbols have the same meanings as before. The upper limit of integration (the threshold) is, however, $h = \alpha V^2$, where α is a constant and V is the voltage at the utilization time. This gives for direct current stimuli and condenser stimuli, respectively, the relations,

$$\log \frac{V}{V - R} = kt + \log \left(\frac{K + k\alpha}{K} \right) \quad (8)$$

and

$$\frac{V}{R} = (\sigma k)^{\frac{1}{\sigma k - 1}} \quad (9)$$

These equations in which an exponential decay of the inadequate excitatory state is implied represent the data very adequately. Therefore, as mentioned above, both the direct evidence for latent addition and the indirect evidence from the strength-duration curves indicate the existence of the exponential rather than the constant decay of Weiss's law.

Considering (9), the condenser equation, first it will be seen that

* This has been written $/ = \alpha /$ previously because some data required the + sign. The cases requiring this sign are so uncommon, however, that they may be due to experimental errors.

when $crk \ll 1$, $\frac{V}{R} = \frac{1}{crk}$, approximately, or $cV = \text{constant}$. The prediction is, then, that the quantity of electricity necessary for condenser stimulation approaches a constant value as the capacity becomes small. In the case of the frog's sciatic nerve, $k = 1000$, approximately, so that if $cr = 10^{-5}$ seconds, approximately, $crk = 0.01$, in which case the exponent of equation (9) $= \frac{1}{0.99} = 1$, approximately. Therefore the quantity of electricity, cV , should be constant, approximately, for time constants, cr , of the order of 10^{-5} seconds and less with this tissue.

That this result is obtained experimentally is illustrated by the work of Scott (1934). He plotted condenser curves as $\log V$ against $\log c$ in which case $cV = \text{constant}$ appears as a line of slope, -1 . His data on the frog's sciatic show cV to be constant in close approximation for voltages from about 10 to 100 rheobases, or as it is remarked,³ for time constants, cr , shorter than 2×10^{-5} seconds.

These results indicate that equation (9), which has been shown previously to represent the data adequately (Blair, 1932 *b*, *d*, 1934, 1935 *c*) up to about 20 rheobases, is valid to about 100 rheobases, at least. They explain also why a formula such as Hoorweg's, which predicts a constant quantity at the limit as the capacity diminishes will fit at least part of the voltage capacity curve.

Hoorweg's law, in common with Weiss's, predicts an energy minimum at $V = 2R$. It appears to be assumed generally that this is borne out experimentally. Equation (9) will be examined therefore in this regard.

Since the energy is proportional to cV^2 , equation (9) is written, after squaring both sides and multiplying by c ,

$$\frac{crkV^2}{R^2} = (crk)^{\frac{1}{crk-1}} = \text{constant} \times E \quad (10)$$

E being the energy. On differentiating and putting in the condition for a minimum,

$$\frac{(crk + 1)(crk - 1)}{2 crk} = \log crk$$

³ Scott, D., *J. Physiol.* 1934, 82, 325

But this is true in the limit as crk approaches 1. But as crk approaches 1, the right hand of (9) approaches $e = 2.718$ etc., the base of the natural logarithms. Therefore according to (9) the energy is a minimum not when $V = 2R$, but when $V = 2.718R$.

Those data which have been shown to conform to equation (9) (Blair, 1932 *b*, *d*, 1934, 1935 *c*) in close approximation will have the minimal energy at this point to a similar approximation. Since this matter has not been discussed previously, however, there is plotted in Fig 1 a group of examples from the data of several observers on several different tissues using condenser stimuli. Only a few of these data have been related previously to equation (9).

In each case of Fig 1 there is plotted the energy⁴ of the discharge against the initial voltage of the condenser on a scale of rheobases. The use of this scale requires that the rheobase should have been given. Some of the older data such as Hoorweg's (1892) do not satisfy this condition, so they could not be used. The scale of energies in the figure is arbitrary, the value, 1, being given to a point at about 1.5 rheobases so that the curve will be well defined in the region around 2 to 3 rheobases. The legend gives the sources of the data and the types of tissues.

It will be observed that in accordance with equation (9) the minima of these curves are close to the points e rheobases which are marked on the graph by short lines. The energy curves are so flat toward the higher voltages in many cases, however, that the minima are poorly defined and the best that can be said, sometimes, is that they occur from about 2.5 to 3 rheobases. Obviously they are not near to 2 rheobases as predicted by Hoorweg's law and as is usually assumed.

In regard to the quantity of electricity with direct currents it will be seen that according to (8),

$$V = \frac{-Rc^{kt+C}}{1 - e^{kt+C}} \quad (12)$$

so that as t becomes very small V becomes approximately constant, as long as C is not equal to zero, and Vt , the quantity of electricity, becomes proportional to the time. This is contrary to Weiss's law

⁴ In case O the square of the energy is used because the energy curve is very flat

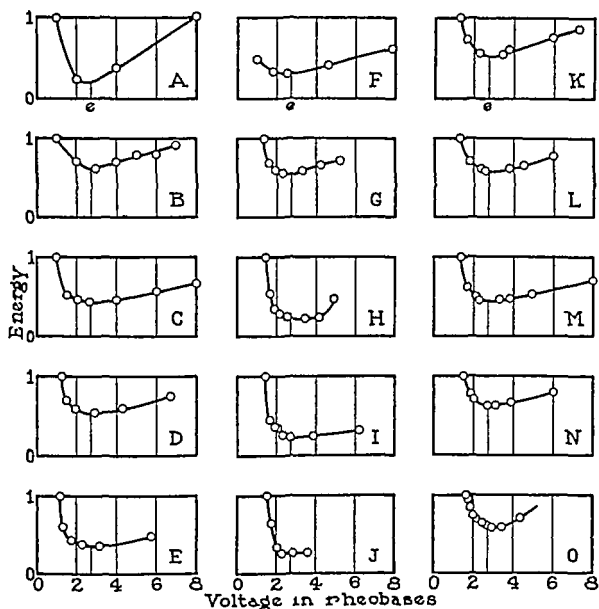


FIG 1 The energy minima for voltage-capacity curves A Sciatic nerve of cat (Waller, 1899 p 217) B Ulnar nerve of man (Waller, 1899, p 218) C Sartorius of toad (Lucas, 1906-07, Experiment 53) D Sciatic tibialis of frog (Hermann, 1906 Experiment 1, p 542) E Sciatic tibialis of frog (Hermann 1906 Experiment 5, p 543) F Sciatic nerve of frog (Lapicque, 1907, 1st set, p 38) G to J Single nerve fibers of sciatic nerve of frog whose impulse velocities are 21.6, 11.6, 11.6, 5.67 and 1.3 meters per second, respectively (E A Blair and Erlanger 1933 given by Blair, 1934, p 127 upper set of fibers Nos 1, 3, 5, 7) K to N Frog's sciatic nerve at various temperatures (Blair, 1935c, p 279 sets 1A, 2A, 3A, and 6A) O Nerve to nictitating membrane of cat, repetitive stimulation (Rosenblueth and Rioch, 1933, Curve A p 523)

but is in accord with experiment, as the curve of quantities plotted against the time tends to go toward the origin instead of cutting the axis of ordinates at the required point (Lapicque, 1926) ⁵

How far this prediction can be taken depends, however, on the dynamics of the process giving rise to C , i.e., to the process lowering the threshold from h to $h - \alpha V$. This process has been complete with the data so far considered in relation to equation (8) but it cannot be assumed that it is instantaneous.

With direct currents on writing equation (8) as,

$$\log \frac{V}{V - R} = kt + C \quad (13)$$

it can be shown similarly that the condition for minimum energy is given by,

$$1 + 2 kt = e^{kt+C}$$

In the special case, $C = 0$, this is true for $kt = 1.25$, approximately, which corresponds to $V = 3.5 R/2.5$, approximately, or about 1.5 rheobases. With the threshold, $h - \alpha V$, C is positive so that kt will be greater than 1.25, and consequently V will be greater than 1.5 rheobases. Therefore, since C is usually positive, the minimum energy will be with voltages somewhat greater than 1.5 rheobases, but its position will be variable, since C is variable and is very seldom zero ⁶. This variability was observed by Lapicque (1926) experi-

⁵ Chapter 5

⁶ A. V. Hill (1932) in reviving Lapicque's (1907) leaky condenser hypothesis, assumes that $C = 0$ in equation (13) (in which case this equation would represent the condenser hypothesis) because of data by Rushton (1932). For these data, this assumption is almost true, but not quite (Blair, 1932c). These data, taken from the frog's sciatic nerve near 0°C are unusual in this respect, however. Most direct current data are not representable by the leaky condenser hypothesis with constant charge for adequacy, even in rough approximation (Blair, 1932a, c, 1935a, b). It should be observed also that equation (9) is true both for this condenser hypothesis and for the hypothesis of equation (7) along with the threshold, $h - \alpha V$ (Blair, 1934). Equation (9) cannot decide, therefore, between the condenser hypothesis and the present one. This ambiguity does not arise with other stimuli, however, which leave the inadequacy of the condenser hypothesis in no doubt as Lapicque (1907) concluded long ago. Hill's (1935) conclusion that an equation like (13) with $C = 0$ must fit direct current data because equation (9)

mentally, who remarks that his results with direct currents did not give minima at the same place consistently. It is evident now why this result was obtained. The situation is illustrated in Fig 2, in

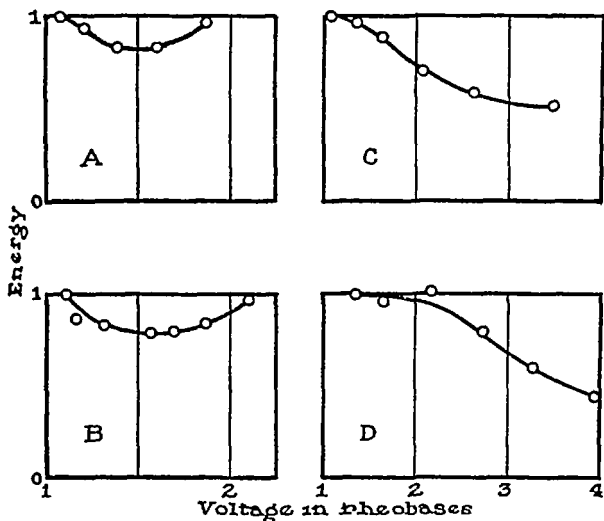


FIG 2 The energy minima with strength duration curves. A Sciatic nerve of frog, $C = 0$ (Blair 1935a, p 761). B Sciatic nerve of frog, $C = 0.03$ (Blair, 1935a, p 761). C Sartorius muscle of frog, $C = 0.12$ (Benoit 1934 p 339). D Sartorius muscle of frog, $C = 0.103$ (Benoit 1934 p 339 Experiments 1 and 3 or Blair 1935d p 304).

which four direct current curves are plotted with the energy as ordinates against the voltage in rheobases as abscissae. Curve A is

fits condenser data appears superficially to be quite valid, but it can be invalid and there is little doubt that it is invalid except in a few special cases. As Hill was concerned chiefly with the effects due to currents of relatively long application (accommodation) this conclusion does not affect his general argument. This matter is discussed again more fully later in the text.

from the sciatic nerve of the frog, and $C = 0$. The minimal energy should be at 1.5 rheobases, according to equation (12). Curve B is also from the sciatic nerve of the frog, but C is greater than zero. The minimum should be displaced to a higher voltage. Curves C and D are *alpha* excitabilities in which (as is usual) C is very large. It will be observed that the minimal energy here is at about 4 rheobases, at least. The curves are not extensive enough to get beyond the minima again in order to determine their positions exactly.

Since the minimal energy for rectangular currents is a variable point greater than or equal to 1.5 rheobases while the minimal energy for condenser stimuli is near e (2.718) rheobases, this particular energy can no longer be considered to have a special significance with respect to the strength-duration curves of various kinds of stimuli expressed on scales of rheobases.

The chronaxie is not at or simply related to the minimal energy point, but this is of no particular consequence, except insofar as they are usually supposed to be coincident. As was pointed out previously (Blair, 1932*c*) the direct current chronaxie does not determine uniquely the excitability factor, k , because of the variable parameter, C , of equation (13). With condenser discharges, however, $crk = 2$ when $V = 2R$, $i = e$ for chronaxie, and $crk = 1$ for minimal energy. Either of these points give directly, therefore, the parameter, k , which is inversely proportional to condenser chronaxie. It will be observed, incidentally, that the minimal energy point gives k without the necessity of the rheobase being known.

The effective energy in the case of rectangular currents is the total energy, but with the condenser stimuli the energy actually utilized is less than the total by the amount still remaining in the condenser at the utilization time. The utilization time for the condenser discharges is,

$$t_2 = \frac{cr}{crk - 1} \log crk = cr \log \frac{V}{P} \quad (\text{Blair, 1932*d*)}^1$$

But according to the equation of the discharge of the condenser ($V = V_0 e^{-\frac{t}{\tau}}$) the potential remaining at the utilization time is

$$V = V_0 e^{-\log \frac{V_0}{R}} = R \quad (14)$$

The condenser discharge is utilizable, therefore, until its potential descends to the rheobase. The utilizable energy consequently is

$$0.5 c (V^2 - R^2) \quad (15)$$

This deduction from the theory can be easily verified directly as follows. The rheobase suffices on long application just to raise the local excitatory state, p , to its threshold. Therefore, if with a greater stimulus, p has not reached the threshold before the potential has dropped to the rheobase it can never do so thereafter. On the other hand, if p has not reached the threshold it will continue to grow as long as the stimulus exceeds the rheobase. Therefore, a just adequate stimulus has raised p just to the threshold just as it itself has decayed to the rheobase. This conclusion was reached by Bouman (1928)⁸ in a similar way. When applied to the equation of the condenser discharge it gives directly the utilization time, the utilizable potential, and the utilizable energy as they are above without reference to any theory of excitation.

This utilizable energy of the condenser discharge (Equation 15) does not present features of particular interest in a simple way so that it need not be discussed further. Equation (14) is of interest, though, with respect to footnote 6, in which it was stated that equation (9) was equally representative of the condenser hypothesis⁹ and the

⁸ P. 421

⁹ Equation (7) has the same form as the differential equation of the rise of potential in a leaky condenser. If the threshold is assumed constant, i.e. if the condenser on reaching a certain potential, h , is assumed to set off the process of conduction the corresponding integral ($C = 0$ in (13)) would represent Lapicque's (1907) condenser hypothesis. This hypothesis is not adequate, but the leaky condenser with a variable threshold ($h = \alpha V$) (C existing in equation (13)) appears to be apart from accommodation. It cannot be inferred, however, on these grounds, that the excitatory mechanism is a leaky condenser with a variable threshold because equation (7) may represent other mechanisms.

present one. It will be evident that with the condenser stimuli, since the excitatory state always becomes adequate when $V = R$, the threshold will always at that moment be $h - \alpha R$ which is a constant. Thus with the condenser discharges the threshold is constant, not because it is unaffected by the stimuli but because it is equally affected by all the stimuli. This is the reason why Equation (9) cannot distinguish between the condenser hypothesis, which assumes a constant threshold, and the present one which assumes a threshold $h - \alpha V$. The threshold for each hypothesis is a constant, although a different constant.

This is the reason also that the condenser equation (9) has but one arbitrary constant, k , while the direct current equation, (8) or (13), has the two, k and C . The second factor, C , is required to express the relations arising from the circumstance that the threshold, $h - \alpha V$, is different for each direct current stimulus because each stimulus has a different value at the utilization time.

It will be evident also that this is the basic reason that condenser chronaxie and direct current chronaxie do not have the same meaning. The former is adequate because it can express a relation determining the single parameter, k , while the latter is not, because it cannot express the two independent parameters, k and C (Blair, 1932 c). The variability of the minimal energy with direct currents is related to the existence of C as was shown above, therefore it also is fundamentally due to a variable direct current threshold.

Thus it will be seen that these differences between the integral relations for the direct current and the condenser stimuli are easily explainable quite directly on the basis of the hypothesis that the threshold is not a constant but of the form, $h - \alpha V$. This hypothesis is well confirmed because it has been shown to be consistent for direct currents, and linearly rising currents (1935 b), in both of which cases the threshold should be variable at the utilization time because each stimulus has a different value at that time, and it is confirmed also by the fact that a constant threshold appears with condenser stimuli, in which case $h - \alpha V$ should be constant.

This alteration of the threshold by the stimulus is not the process of accommodation the dynamics of which have been discussed recently again by Hill (1935). Accommodation involves a raising of the

threshold, among other things, and is a slow process compared to the excitatory process proper, so slow that its effect on the strength duration curve is apparently negligible (Blair, 1935 *b*) The present process on the other hand is very fast relative to the excitatory process and it involves a lowering of the threshold?

It appears to be well established, therefore, that there are two excitatory processes apart from accommodation. The principal one is that represented by the equation,

$$\frac{dp}{dt} = KV - kp$$

The other one, whose dynamics are not known because it is always complete with existing data at the utilization time, involves a lowering of the threshold from h to $h - \alpha V$. There is some indication from unpublished data on the *alpha* excitability that this threshold process is not complete with the very short stimuli, so its dynamics may be found in this way. The question of its existence does not depend, however, on a knowledge of its dynamics.

CONCLUSIONS

It will be evident, in conclusion, that the quantity of electricity and the energy do not appear from excitation data in a way nearly so simple as has been generally supposed on the basis of Weiss's and Hoorweg's laws and that in consequence the attachment of any special significance to these factors is likely to be very misleading. The situation given by the data is explainable quite directly on the basis of the hypothesis that the rate of growth of the excitatory state varies directly as the instantaneous strength of the stimulus, that there is a simultaneous subsidence of the excitatory state at a rate proportional to its magnitude, and that the threshold amount of the excitatory state is a constant decreased by an amount proportional to the strength of the stimulus at the utilization time. The present considerations, therefore, lend further support to this hypothesis.

SUMMARY

Weiss's and Hoorweg's laws are discussed with respect to the dynamics of the excitatory process. The former is shown to have a simple basis which is inadequate, however, because it implies a con

stant rate of subsidence of the state of excitation. Hoorweg's law does not follow logically from the same basis so the two laws do not represent the same excitatory mechanism. Experimental data do not give minimal energies at 2 rheobases as predicted by each law. The experimental minima with direct currents are at 1.5 or more rheobases, while with condenser stimuli they are from 2.5 to 3.0 rheobases. These minima conform to the predictions of the writer's equations which give the direct current minima as variable with a lower limit at 1.5 rheobases and the condenser minima as constant at $c = 2.718$ rheobases. The reasons for these differences are discussed and it is concluded that considerations of the quantity of electricity and the energy, *per se*, do not lead to any simple concepts with regard to the excitatory mechanism. The existing quantity and energy relations are, however, easily correlated in terms of the dynamics of the excitatory mechanism.

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INTERMITTENT STIMULATION BY LIGHT

V THE RELATION BETWEEN INTENSITY AND CRITICAL FREQUENCY FOR DIFFERENT PARTS OF THE SPECTRUM*

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I

Expected Results

Our previous studies of the relation between light intensity and critical fusion frequency (Hecht and Verrijp, 1933 b) have shown that the differences which the measurements exhibit when they are made in different retinal locations are an expression of the duplex structure of the retina (Schultze, 1866, Parnaud, 1885, von Kries, 1929). With a centrally located 2° field the data are continuous over the whole intensity scale, and may be described by a simple sigmoid curve, whereas with a peripherally located 2° field the data divide sharply into a low intensity section and a high intensity section each of which may be described by a single curve. Since the central, 2° field falls within the rod free area of the retina, the continuous nature of the data indicates that they are a function of the cones alone. The double nature of the peripheral measurements very likely represents rod function for the low intensity section and cone function for the high intensity section. This is borne out by the increasing separation of the two sections as measurements are made farther and farther from the center: the cone section shifts to higher intensities and the rod section to lower intensities, as would be expected from the increasing ratio of rods to cones in these regions.

In order to confirm the identification of the two sections with rod

* A preliminary report of these measurements was made to the Optical Society of America in February 1935 (Hecht and Shlaer 1935) and to the XV International Physiological Congress in Leningrad, in August, 1935.

and cone function, we have now used different parts of the spectrum to study the relation of critical frequency to intensity. For this purpose we employed a central retinal area 19° in diameter, containing both rods and cones.

Fig 1 gives the relative spectral sensibilities of the cones and rods, and shows what may be expected of the measurements. Spectral

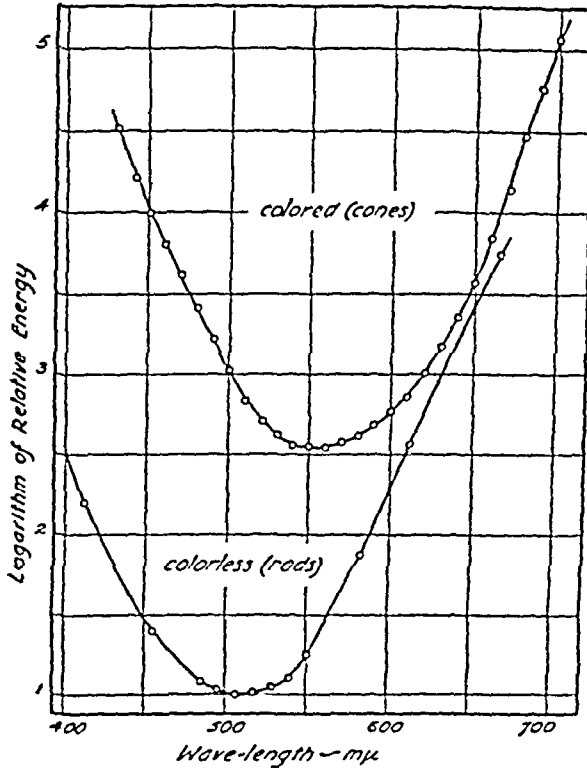


FIG 1 Relative spectral sensibilities of the rods and cones. The curves are each accurately drawn: the cone curve is from the data of Hyde, Iorsythe, and Cadz (1918), and the rod curve from the data of Hecht and Williams (1922). The vertical separation of the two curves is arbitrary and conforms to the fact that the colorless and color thresholds of the eye are nearly coincident in the red.

energy can produce no visual effect until it reaches the relative intensity indicated by the rod curve. Above that, rod function dominates until the cone threshold is reached. The intensity distance over which the rods dominate in visual function changes throughout the spectrum: between 670 and 630 $m\mu$ it is small and alters only slowly, beginning at about 600 $m\mu$ and going toward the blue the dis-

tance becomes rapidly larger, while below 500 $m\mu$ it remains practically constant

Preliminary investigation (Hecht and Verrijp, 1933*a*) confirmed these expectations. Critical frequency measurements with a small peripheral field showed that whereas the high intensity portions are much the same for all colors, the low intensity section is very short with red light and becomes longer as the light moves down the spectrum toward the blue. The monochromatic filters used by Hecht and Verrijp reduce the brightness to about 1 per cent of the incident light. With the optical system in the apparatus (Hecht, Schlaer, and Verrijp, 1933), this reduction limited the measurements to only a part of the high intensity cone curve. We have therefore redesigned the optical system so as to furnish about 100 times as much light as in the previous research. At the same time we have increased the total field size to a diameter of 35° which may be used in various configurations of non flickering surround and flickering test area.

II

Apparatus and Procedure

Fig 2 shows the new arrangements. An image of the incandescent ball of a 2 ampere Osram Punktlicht is focussed by lens $L1$ on the plane of the sector disc. The diverging light is then converged by $L2$ through the unsilvered portion of the photometer cube to fill the field lens $L3$. This forms the flickering central field. Another image of the same source is made to fill the silvered section of the photometer cube in an analogous way by lenses $L1'$ and $L2'$. This constitutes the non flickering surround. The surround is made equal in brightness to the flickering field (above the critical fusion frequency) by the movement of lens $L2'$, which controls the divergence of the beam in filling $L3$. $L3$ then focusses the two images of S_1 and S_1 on the field lens $L4$ of the viewing telescope which in turn forms an image of the photometer cube in the focal plane of the ocular of the telescope. Finally, the ocular focusses images S_2 and S_2' of the source on the pupil of the eye. The size of the final combined image of the source, as it appears in front of the ocular and falls on the pupil of the eye, is 1.5×1.3 mm. There is thus no necessity for an artificial pupil in this system, since the image size remains constant and well below the smallest pupil at the highest brightnesses.

Before it enters the telescope objective the light passes through the circular unsilvered portion of a small photometer cube C , the silvered portion of which reflects a fixation point into the field. The fixation point consists of a pin hole P , strongly illuminated by the image of a flashlight lamp filament projected by

a microscope objective. The pin hole is at such a distance from the telescope as to be in focus at the same time as the image of the large photometer cube. The fixation point is mounted as a unit with its lamp and objective, and is movable in all directions within the optical field. The brightness of the fixation point is controlled by a potentiometer available to the observer.

Between the field lens $L3$ and the telescope objective $L4$, there are neutral and monochromatic filters and a neutral wedge and balancer for intensity and color control. We used two neutral filters transmitting approximately 1/100 and

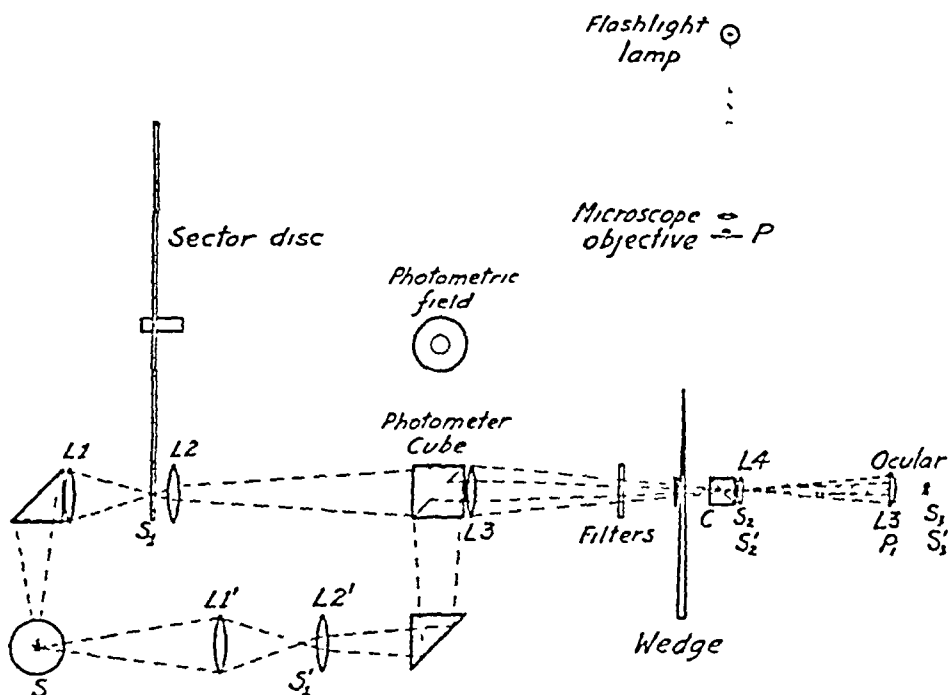


FIG. 2. Diagram of the new optical arrangements for flicker measurements.

1/10,000. The wedge and neutral filters were calibrated separately for each of the color filters with a Martens polarization photometer as already described in paper II of this series (Hecht, Schlaer, and Verriyp, 1933).

Because of the ocular, the absolute brightness had to be determined by a binocular match between the test field as viewed with the right eye, and a fixed brightness viewed through a 2 mm diameter pupil with the left eye. After measuring with a Macbeth illuminometer the brightness of the surface viewed through the left eye, and taking the artificial pupil size of the left eye into consideration, the brightness as seen with the right eye may be described in terms of photons¹

The procedure for making the measurements was almost the same as described in the second paper of this series with two changes added as a result of our experience. In all cases now, the intensity was set and the frequency varied by a slide rheostat until flicker disappeared. Two readings at each intensity were usually adequate; if they did not agree closely we made a third and rarely a fourth. As before care was taken to secure complete adaptation to each intensity, but now we allowed no rests in the dark between intensities. We found the rests in the dark neither necessary nor beneficial and without them we could maintain a continuous state of light adaptation which easily changed its level with increasing intensities. By this procedure we were able in 2 hours to span the whole visible intensity range, making from 16 steps in the red to 24 steps in the blue.

In all the measurements to be reported in this paper, we used a circular test field 19° in diameter; it was always surrounded by a non-flickering circular area 35° in diameter, of the same brightness as the test field when it is interrupted at rates beyond the critical fusion frequency. The intensities given are for the non-flickering central test field, and therefore are twice the brightness of the surround.

III

RESULTS

The results are given in Table I. Each datum is the average of the measurements made in three separate runs with the right eye of each

¹ The photon as a unit of retinal illumination was suggested by Troland (1916). Intensities measured in millilamberts are converted into photons by multiplying with the factor $10a/\pi$, where a is the effective area of the pupil. In Paper II of this series (Hecht, Shlaer, and Verrijp, 1933), we misinterpreted Troland's definition of photon and made an error in this conversion factor. Because of this and of another minor error, it is necessary to multiply by 40 the numbers given as photons in the three preceding papers of this series (Hecht, Shlaer, and Verrijp 1933, Hecht and Verrijp, 1933*b* and *c*) in order to make them comparable with the intensities in this and in the following paper by Hecht and Smith.

As an expression of actual retinal brightness, the photon is obviously superior to the millilambert which gives intensities external to the pupil. Nevertheless photons for different pupil openings record identical retinal brightness only when the pupil areas are small, say below 2.5 mm in diameter (cf. Stiles and Crawford 1933).

of us. The table shows that as the spectrum changes from red to blue, measurements of critical frequency become possible at lower and lower intensities. This is what we had anticipated from the spectral sensibility distribution for the rods and cones.

The information conveyed by the measurements can best be understood from their graphic representation. As Fig. 3 (the data are for S. H.) shows, the data break into two sections. The high intensity

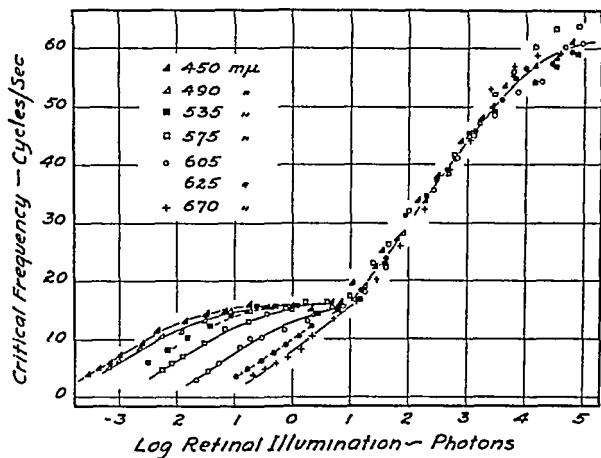


FIG. 3. The data of S. H. showing the relation of critical frequency to $\log I$ for the different spectral regions shown.

portions, which we have identified with cone function, fall together for all the colors. We have graphically superimposed the various cone data on each other, and have compared the results with the superposition achieved on the basis of heterochromic photometry. Only the slightest differences appear between the two methods, the differences being haphazard and well within the errors both of heterochromic matching and of our superposition judgments. The low intensity sections, which we have identified with rod function, are

spread out much as expected, and extend to lower and lower intensities with decreasing wave-length

Fig 3 resolves the mystery of Ives' old findings (Ives, 1912) that the low intensity portions of critical frequency data for different parts of the spectrum may be represented by straight lines which differ in slope, the red being steepest and the violet being practically horizontal

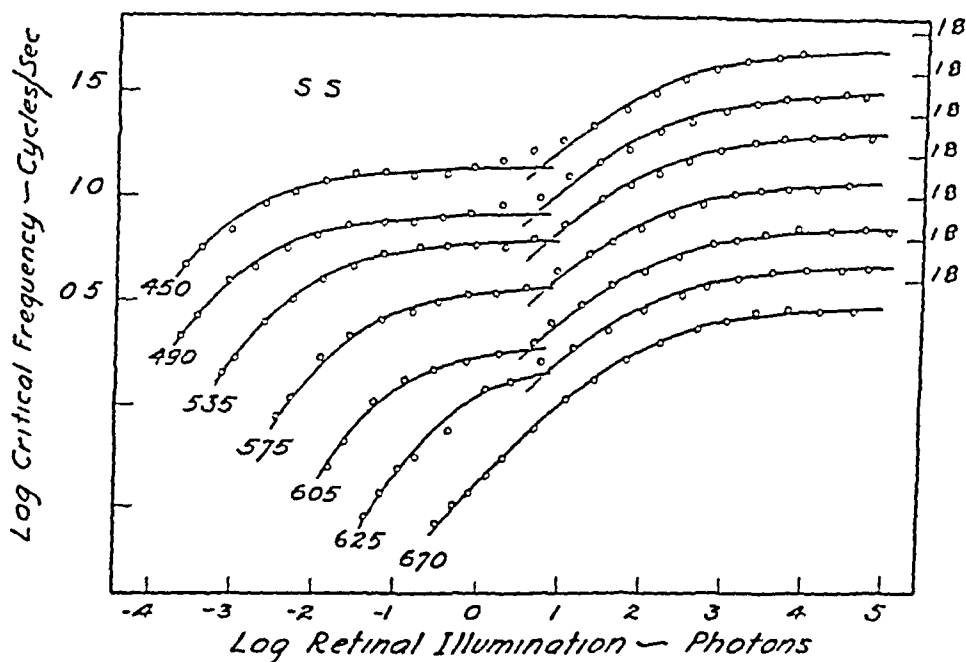


FIG 4 The data of S S plotted as log frequency against log I for the different spectral regions. The numbers on the ordinates to the left apply to the topmost data alone, for convenience the other data have been moved down in steps of 0.2 log unit, and their exact positions are indicated to the right. The curves are from equation (1) for the high intensity cone portions, and from equation (2) for the low intensity rod portions.

It is apparent in Fig 3 that for short stretches near the rod-cone transition, straight lines can be drawn through the rod data, showing different slopes for the different wave-lengths.

The real phenomenon, however, is something quite different. It is that the separation of rod and cone sections as a whole increases as the wave-length decreases. This is shown strikingly by Fig 4 in

which the data of S S are plotted as the logarithm of the critical frequency against the logarithm of the intensity. The data for $670\text{ m}\mu$ fall on a single, continuous curve, whereas the data for all other parts of the spectrum are best described by two separate curves. The high intensity curve is in the same position for all colors, and the only effect of changing the spectral composition of the light is to shift the position of the low intensity, rod curve along the intensity axis, without in the least changing its form. From our first measurements (Hecht and Shlaer, 1935) we were inclined to believe that the rod curve shifts along the vertical axis as well. However, the average data of S H show no vertical shift at all, while those of S S show a slight displacement which is sufficiently haphazard and small to be neglected as within the range of variation.

The transition between rod and cone portions is quite sharp for all but the blue and violet data. On either side of the transition, the identification of rod function and cone function is borne out by subjective observation. The test field, being 19° in diameter, contains the whole macula as well as periphery. At low intensities and below the critical fusion frequency the flicker is distinctly located in the peripheral portion of this field, so that the field resembles a flickering doughnut. As the critical frequency is approached, the last appearance of flicker is always in the periphery. With increasing intensity, the first sign of approaching cone function is the appearance of color in the field, which becomes identifiable with certainty about 0.5 log unit below the actual inflection point of the measurements. At the intensities around the transition and near the critical frequency, two separate centers of flicker are very often apparent, one in the periphery and the other in the center, and it is difficult to predict which will disappear later and thus determine the critical fusion frequency. As a result, this is a region of difficult measurement, and of daily variation. At intensities higher than the transition intensity but near it, flicker usually persists longest in the center, but beyond these intensities the last trace of flicker may be in any part of the field. Obviously the rods determine the low intensity section, and the cones the high intensity section, but the specific cones which set the critical frequency are not necessarily the same throughout the high intensity section.

IV

Theory

The curve which in Fig 4 is drawn through the data for 670 m μ represents the equation

$$KI = f^2/(f_{max} - f)^2 \quad (1)$$

in which I is the intensity and f the critical fusion frequency K is a constant, which in a log I -log f plot determines the position of the curve on the intensity axis, just as f_{max} determines its position on the vertical frequency axis, neither K nor f_{max} having any influence on the shape of the curve from the equation. It is apparent that the equation describes the entire 670 m μ data with precision. The same curve has been drawn through the cone portions of all the other parts of the spectrum, even though it slights the transition points for the blue and violet. The rod portion of all the measurements has the curve drawn through it from equation

$$KI = f/(f_{max} - f)^2 \quad (2)$$

in which the symbols have the same meaning as before

Although equations (1) and (2) may be considered as purely empirical expressions to describe the data, they nevertheless can be derived from the familiar reversible photochemical system which has been useful in describing many aspects of vision and the photosensory process (Hecht, 1934). Generalizing the derivation previously made (Hecht and Wolf, 1932, Hecht and Verriyp, 1933 *c*), the steady state, when the light and dark periods of the intermittent illumination are of equal duration, may be written as

$$KI = x^n/(a - x)^n \quad (3)$$

where n' and n are the reaction orders of the photochemical and dark reactions respectively

If we make the critical frequency f proportional to the concentration x of photoproducts at the steady state, then equation (3) becomes the flicker equation (1) for the cones, provided $n' = n = 2$, as shown by the data of intensity discrimination (Hecht 1935). Similarly, when $n = 2$ and $n' = 1$, equation (3) becomes the same as (2) used for describing the rod data

The assumption (Hecht and Vernip, 1933 *c*) that f is proportional to x^2 has to be discarded because it requires the intensity factor to enter the equations as I^2 , and Arnold and Winsor (1934) have definitely shown that I must enter as the first power if Talbot's law is to hold. This change introduces nothing new for the rods since the rod dark reaction at the steady state is also proportional to x ($n = 1$) and the resulting equation (2) is almost identical with the old one (Hecht and Vernip, 1933 *c*). For the cones, however, the data persist in showing two contradictory things. According to theory, the critical frequency should be proportional to the velocity of the dark reaction. The data clearly show (see particularly the following paper) that for the dark reaction $n = 2$, so that f should be proportional to x^2 . Yet the data follow equation (1) only when f is made proportional to x .

One way of resolving this contradiction is to suppose that the proportionality of f to x indicates the dependence of the critical frequency for the cones not on the dark reaction which reforms the sensitive material, but on the secondary dark reaction which follows the photochemical one in time and which uses the photoproducts to form impulses that leave the cell. There is no reason to suppose that the velocity of this reaction is anything but directly proportional to the concentration of photoproducts rather than to their square.

SUMMARY

1 An optical system is described which furnishes large flickering fields whose brightness, even when reduced with monochromatic filters, is capable of covering the complete range of the relation between critical frequency and intensity.

2 For a centrally located test field of 19° diameter, with light from different parts of the spectrum, the data divide into a low intensity section identified with rod function, and a high intensity section identified with cone function. The transition between the two sections is marked by an inflection point which is sharp, except for 450 and 490 $m\mu$ where, though clearly present, it is somewhat rounded.

3 The intensity range covered by the flicker function is smallest in the red, and increases steadily as the wave length decreases. The increase is due entirely to the extent of the low intensity, rod section which is smallest (non-existent for S-S) in the red and largest in the

violet The high intensity cone portion for all colors is in the same position on the intensity axis, and the only effect of decreasing wavelength is to shift the rod section to lower intensities without changing its shape

4 The measurements are faithfully described by two similar equations, one for the rods and one for the cones, both equations being derived from the general stationary state equation already used for various visual functions

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INTERMITTENT STIMULATION BY LIGHT

VI AREA AND THE RELATION BETWEEN CRITICAL FREQUENCY AND INTENSITY*

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I

For a central area approximately 2° in diameter the retina is practically rod free and contains only cones. Outside of this area, rods appear and increase in number toward the periphery. Judging by this fact and by previous work on flicker (Hecht and Verrijp, 1933), on intensity discrimination (Steinhardt, in press, cf. Hecht, 1934), and on dark adaptation (Hecht, Haig, and Wald, 1935), the relation between critical fusion frequency and intensity as measured with central areas smaller than 2° in diameter should be a continuous function representing cones, whereas with larger areas the relation should show a duplex character illustrative of the predominant working of rods at low intensities, and of cones at high intensities.

We have measured the relation between critical fusion frequency and intensity for white light, using four centrally located areas 0.3° , 2° , 6° , and 19° in diameter, and our measurements confirm these expectations.

II

In making the measurements we used the apparatus described by Hecht, Shlaer, and Verrijp (1933) with the new optical system and procedure described in the immediately preceding paper of this series by Hecht and Shlaer. The four sizes of test field were obtained with four separate photometric cubes having the corresponding openings.

* A preliminary report of this work was given to the Optical Society of America in February, 1935 (Hecht and Smith 1935) and to the XV International Physiological Congress in Leningrad in August, 1935.

in the silvering on the diagonal face The holes in the silvering are actually ellipses, but in front view they appear circular

The surround for all the test fields has the same diameter, 35° This increase in size of surround in comparison with the 10° one used earlier was for the purpose of removing, if possible, the drop in critical frequency which occurs at very high intensities after the critical

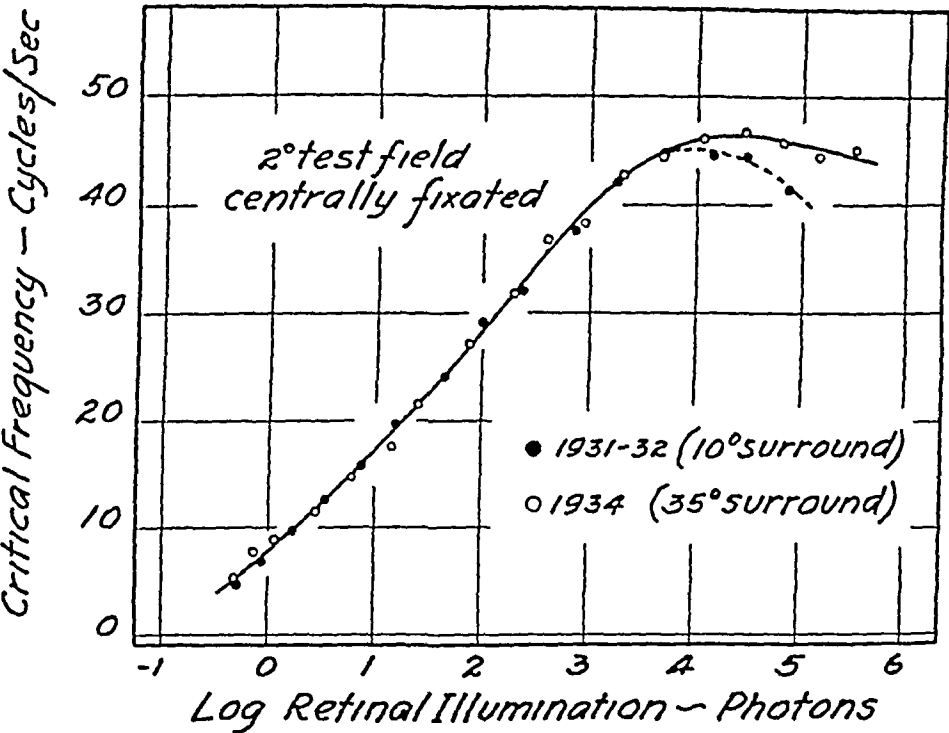


FIG 1 Relation of critical frequency to retinal illumination Comparison of previous measurements with a 2° centrally fixated field made in 1931 and 1932 (Hecht and Verrijp, 1933) with a 10° surround, and repeated in 1934 with a 35° surround Right eye of S H

frequency has reached a maximum Since the drop is prominent in the absence of a surround, it seemed likely that a surround larger than 10° would eliminate most of the comparatively small drop previously found with the 10° surround

Fig 1 shows the data of S H with the 2° test field, using the new surround of 35° and the old one of 10° The two sets of data are practically identical except at high intensities where the new observations

show only a slight decrease in critical frequency beyond the maximum. Even this decrease is frequently absent, again and again in these and in the preceding measurements with color, we have made runs in which the top of the curve is entirely flat.

The importance of a large surround in this type of measurement is also evidenced by its subjective effects. The glare and discomfort, and even subsequent headaches, characteristic of working at very high intensities with a small field minus a surround, are much mitigated even with a 10° surround, and are almost completely absent with the 35° surround.

III

The data are given in two tables. Each datum represents the average of three complete runs, in each run two observations were usually made at each intensity, though on the infrequent occasions when these did not agree closely, three and four measurements were included. The tables show that as the field size increases, measurements of critical frequency can be made at lower and lower intensities.

The meaning of the data is apparent from Fig. 2, where the measurements of E. L. S. are plotted in the usual manner of critical frequency against $\log I$. As expected, the measurements for 6° and 19° break into two sections which from previous work must be identified with rod function at low intensities and with cone function at high intensities. Note that the rod part is less extensive, and its plateau lower for the 6° field than for the 19° field. For the 2° and 0.3° fields the rod part is definitely missing.

For E. L. S. the maximum critical frequency at high intensities increases with field size. The data for S. H. show only slight differences in maximum for the different areas, in fact, the 6° field is actually slightly higher than the 19° field. No great reliance is to be placed on this because of the comparatively large variation in maximum shown by S. H. in the course of the measurements.¹

The behavior of the low intensity rod section of the data with

¹ The slope of the curve relating frequency to $\log I$ undergoes similar variations since the slope is determined by the maximum (or the reverse), and depends to a large extent on the criterion adopted for the critical frequency as well as on unexplained daily and long time variations (*cf.* Hecht and Verrijp 1933 footnote).

increasing area resembles its behavior with a 2° test field placed in different retinal locations. The farther in the periphery the 2° field is measured, the lower is the position of the rod section on the in-

TABLE I

Brightness and Critical Fusion Frequency for Circular Areas of Different Diameter, Centrally Fixated Data for S II

Intensity in photons	Cycles per second			
	0.3°	2°	6°	19°
0 00120				3 48
0 00182				4 61
0 00275				5 42
0 00407				6 71
0 00933				8 07
0 0174			2 94	9 29
0 0398			4 91	10 5
0 0891			6 41	10 9
0 200			7 40	11 8
0 468		5 33	8 52	11 8
0 724		7 69		
1 12	6 38	8 84	9 46	11 6
1 82	9 03			
2 75	9 87	11 6	11 2	11 6
6 17	13 1	14 7	14 1	13 6
14 1	16 9	17 6	17 5	15 9
31 6	20 4	21 4	21 4	23 0
75 9	27 1	27 2	26 4	27 5
191	27 7	31 7	32 2	33 2
398	33 1	36 7	36 9	36 3
891	36 8	38 2	41 4	39 7
2000	41 3	42 8	47 5	42 5
4680	44 1	44 4	49 3	42 9
11200	48 0	46 2	51 6	43 3
27500	49 6	46 7	52 3	46 8
61700	49 0	45 7	52 3	47 1
141000	47 4	44 7	53 4	48 1
316000		45 0	53 7	49 7

tensity axis (Hecht and Verrijp, 1933). The same is true in the present data when the centrally fixated area is increased. It is as if the effect of increasing the area is mainly concerned with bringing the measurements into the periphery. A similar situation exists in rod dark adaptation where the increasing adaptation range associated with

increasing test fields is duplicated by a small test field placed in increasingly more peripheral locations (Hecht, Haig, and Wald, 1935)

TABLE II

Brightness and Critical Fusion Frequency for Circular Areas of Different Diameter, Centrally Fixated Data for E. L. S.

Intensity in photons	Cycles per second			
	0.3	2	6	19*
0 00120				2 95
0 00182				4 13
0 00275				5 87
0 00407				7 18
0 00933				9 11
0 0174			4 86	11 7
0 0398			5 90	12 9
0 0891			7 38	14 3
0 200		2 45	9 18	15 7
0 302		3 68		
0 468		5 56	9 69	16 0
0 724		7 10		
1 12	6 18	10 2	10 3	15 8
1 82	8 58			
2 75	10 2	13 8	12 8	16 0
6 17	13 4	16 0	15 5	20 3
14 1	15 3	19 1	19 2	25 0
31 6	17 6	22 4	24 5	30 1
75 9	19 9	26 9	29 1	33 6
191	22 2	31 1	33 7	37 1
398	26 9	34 6	38 0	42 5
891	30 8	38 2	40 8	45 8
2000	33 7	42 0	43 2	50 4
4680	37 1	43 9	44 6	55 3
11200	39 6	44 3		56 6
13800			45 6	
27500	41 6	44 9		58 1
32400			45 3	
61700	39 2	44 4	44 2	55 4
141000	39 2	44 1		55 0
182000			46 3	
316000	39 4	43 6		56 8

The position of the cone sections of the present data seems to be the same regardless of the area of the centrally fixated test field. The cones in the center of the eye, being the most sensitive,

increasing test fields is duplicated by a small test field placed in increasingly more peripheral locations (Hecht, Haig, and Wald, 1935)

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0 302		3 68		
0 468		5 56	9 69	16 0
0 724		7 10		
1 12	6 18	10 2	10 3	15 8
1 82	8 58			
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6 17	13 4	16 0	15 5	20 3
14 1	15 3	19 1	19 2	25 0
31 6	17 6	22 4	24 5	30 1
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398	26 9	34 6	38 0	42 5
891	30 8	38 2	40 8	45 8
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27500	41 6	44 9		58 1
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61700	39 2	44 4	44 2	55 4
141000	39 2	44 1		55 0
182000			46 3	
316000	39 4	43 6		56 8

The position of the cone sections of the present data seems to be the same regardless of the area of the centrally fixated test field. The cones in the center of the eye, being the most sensitive,

at about the same intensity regardless of whether or not they are surrounded by other, less sensitive cones in the periphery. This is borne out by the measurements of a 2° field placed in different retinal locations (Hecht and Verrijp, 1933) in which the cone section appears at higher intensities the farther the test field is placed in the periphery. Thus the rod section and the cone section of the data behave differently with area and retinal location, depending on the fact that the sensitivity and number of rods increase as one goes toward the periphery.

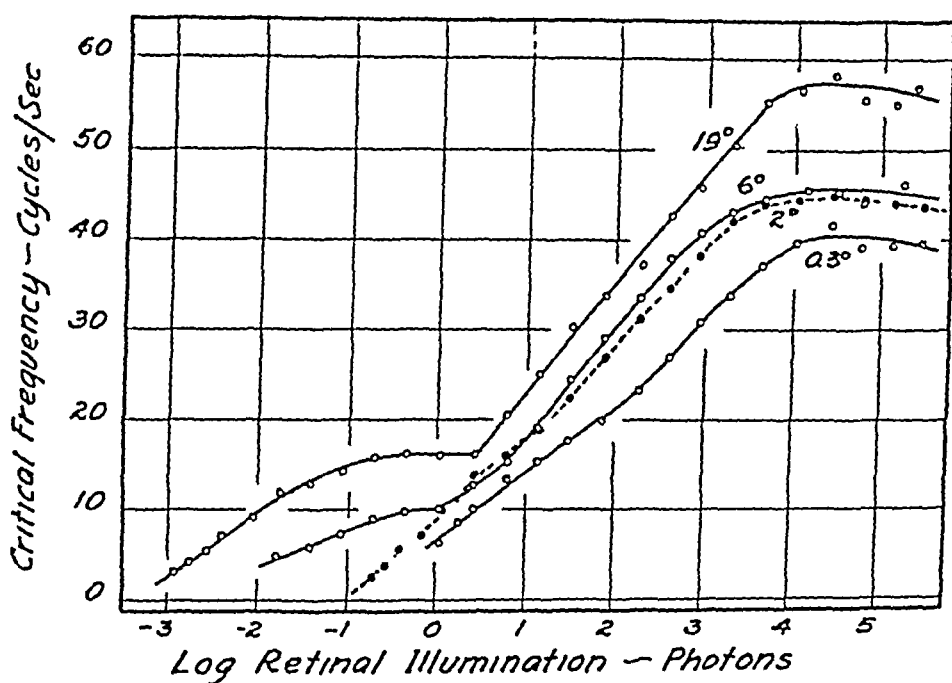


FIG. 2. Influence of area of centrally fixated test field on the relation between critical frequency and $\log I$. Data of E. L. S.

while the sensitivity and number of cones decrease under the same conditions.

The data for the 6° and 2° fields are of pointed interest in the problem of flicker and area. Except for the absence of the rod piece in the smaller field, the two sets of data are almost identical. Under the circumstances of possessing the same surround, a ninefold increase in area of the test field hardly changes the relation of critical frequency to intensity so far as cone function is concerned (cf. Granit and Harper, 1930).

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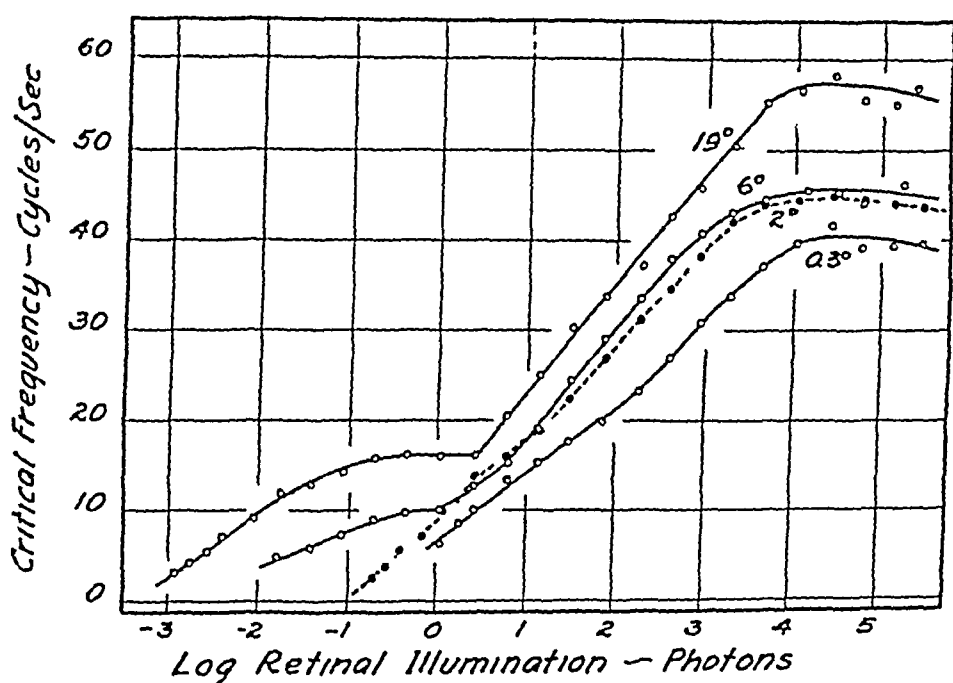


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There is a curious bend in the 0.3° data and to a less extent in the 2° data which we find persistently present in both our measurements. The obvious possibility that this bend represents a rod admixture is excluded on three grounds. First, the bend is more evident in the smaller, central field than in the larger, rods would be more likely to appear in the larger field. Second, the location of the bend is at a different critical frequency (and intensity) from the rod sections of the other data. Third, measurements of a small central field with

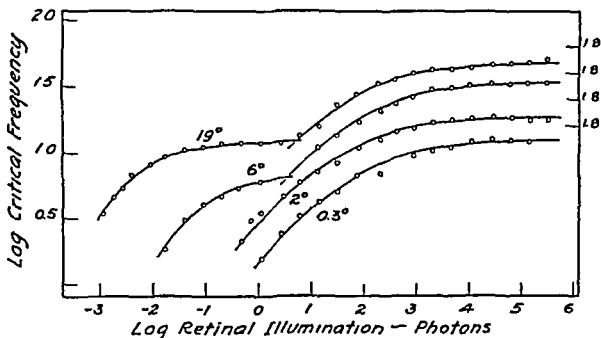


FIG 3 Area and the flicker relation. The log I axis is the same for all the data. The numbers on the log frequency axis to the left apply to the uppermost data only, the other data have been moved down in steps of 0.2 log unit in order to space them, their precise position being given on the right ordinate axis. The curves drawn are from equation (1) for the cone portions and from equation (2) for the rod portions.

white light, red light, and violet light show no differences in the position and magnitude of the bend. As is evident from the preceding paper, this procedure would separate out the function of the rods if they were present. We hope to investigate the nature of the bend later in more detail.

Fig 3 presents the data of S H as the logarithm of the critical frequency (f) against the logarithm of the intensity (I). This type of plot shows more clearly the phenomena already described. In spite of the irregularity in the 0.3° data, a single curve describes the meas-

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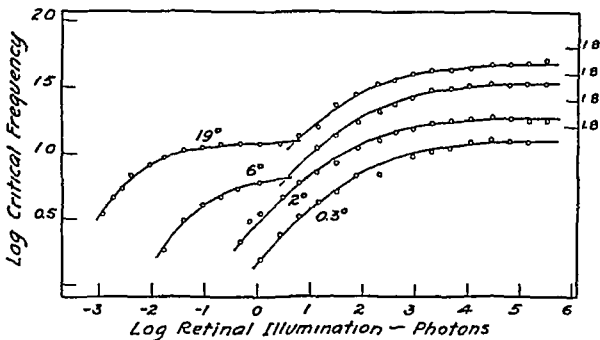


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urements fairly well. The single curve is even more expressive of the 2° data. The curve drawn is the one used for the cones in the preceding measurements on color, and is also drawn through the cone portions of the 19° and 6° data. Its equation is

$$KI = f^2/(f_{max} - f)^2 \quad (1)$$

where K is the constant which determines the position of the curve on the intensity axis, just as the value of f_{max} determines its position on the critical frequency axis.

The rod sections of 19° and 6° require a slightly different curve which is the same for the two fields. Its equation is

$$KI = f/(f_{max} - f) \quad (2)$$

where the terms have the same meaning as before.

It is worth emphasizing that the rod sections of the 19° and 6° fields have the same curve drawn through them. While this is not clearly seen in an ordinary plot of critical fusion frequency against $\log I$, it becomes plain in the $\log f$ - $\log I$ plot of Fig. 3. The identity of the curves shows that the difference between the 19° and 6° rod data is not basic, but merely represents a displacement on the axes in the log plot corresponding to a change in the scale of plotting on the ordinary plot. Exactly the same is true for any systematic differences which the cone data show. Fundamentally the systems in the rods and cones which determine the relation between critical frequency and intensity remain the same regardless of area. Only the dimensional constants are changed by changing the area.

IV

Equations (1) and (2) whose curves have been drawn in Fig. 3 are varieties of the stationary state equation

$$KI = x^n/(a - x)^m \quad (3)$$

in which frequency f is made proportional to concentration of photo-products x , and m and n are the reaction orders of the photochemical and dark reactions respectively. The four varieties of equation (3) corresponding to values of m and n as 1 or 2, are shown in Fig. 4.

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where the terms have the same meaning as before.

It is worth emphasizing that the rod sections of the 19° and 6° fields have the same curve drawn through them. While this is not clearly seen in an ordinary plot of critical fusion frequency against $\log I$, it becomes plain in the $\log f$ - $\log I$ plot of Fig 3. The identity of the curves shows that the difference between the 19° and 6° rod data is not basic, but merely represents a displacement on the axes in the log plot corresponding to a change in the scale of plotting on the ordinary plot. Exactly the same is true for any systematic differences which the cone data show. Fundamentally the systems in the rods and cones which determine the relation between critical frequency and intensity remain the same regardless of area. Only the dimensional constants are changed by changing the area.

IV

Equations (1) and (2) whose curves have been drawn in Fig 3 are varieties of the stationary state equation

$$KI = x^n/(a - x)^m \quad (3)$$

in which frequency f is made proportional to concentration of photo-products x , and m and n are the reaction orders of the photochemical and dark reactions respectively. The four varieties of equation (3) corresponding to values of m and n as 1 or 2, are shown in Fig 4.

Examination of the data in Fig 3 (and of the data in Fig 4 of the preceding paper on color) shows that the rod curve always has twice the slope of the cone curve. This determines the value of n in the two cases, $n = 1$ for the rods, and $n = 2$ for the cones. The best curve to fit the cone data always has $n = 2$, and $m = 2$, as was found also for intensity discrimination (Hecht, 1934). The rods, however, are somewhat variable with regard to the value of m . This is illus-

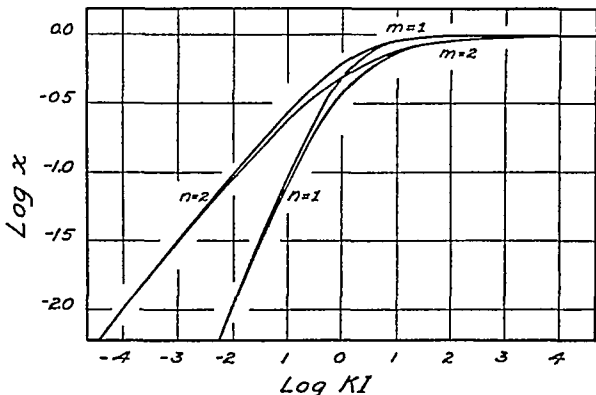


FIG 4 The stationary state equation (3) plotted when m and n are each 1 and 2. Because of the log plot the shape of the curves remains constant regardless of the values of K and σ which merely locate the position of the curves on the axes.

trated by Fig 5 which contains our individual measurements with the 19° field. Besides showing the adequacy and reproducibility of the data, especially in relation to the curves, Fig 5 indicates this systematic variability of the rod measurements. Of the six runs, the rod data of four are described adequately by equation (3) only when $m = 2$, while the two others are better fitted when $m = 1$. Fig 4 shows that when $m = 2$, $n = 1$, the curvature is more gradual, whereas when $m = 1$, $n = 1$, the transition between the rising limb and the plateau is sharper. Also, the plateau itself continues to

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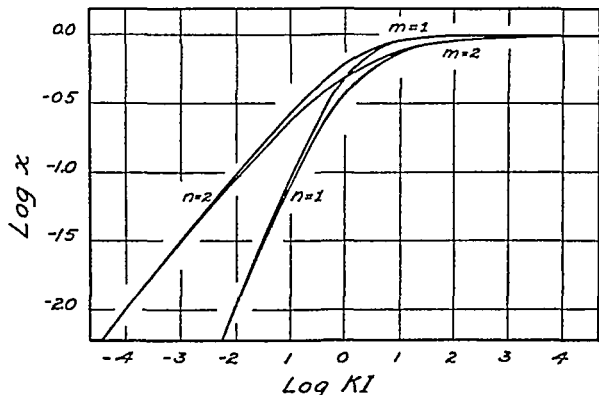


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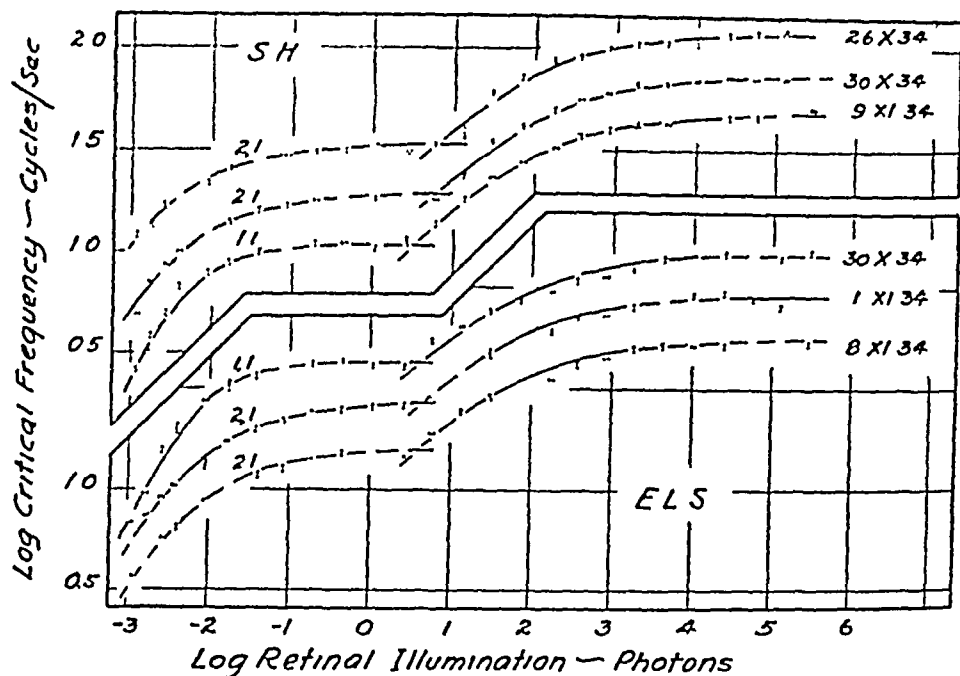


FIG. 5. Critical frequency and retinal illumination for a centrally located 19° field. Each individual measurement as taken in the course of a run is shown as a dot. For convenience, the separate runs (dated to the right) have been spaced 0.2 log unit apart on the vertical axis, the values on the ordinate scale refer only to the topmost run for each investigator. The numbers attached to the rod curves indicate the values of m and n in equation (3) used in drawing them.

sistency with which either one or the other type of curve appears is, however, impressive for us who have watched them for many months.

SUMMARY

1. In the retina, central areas whose diameter is less than 2° possess only cones, while larger areas have rods and cones. In conformity with this, the relation of critical fusion frequency to intensity is a single function for centrally fixated areas below 2° , and a double function for similarly fixated, larger areas. The two sections of such

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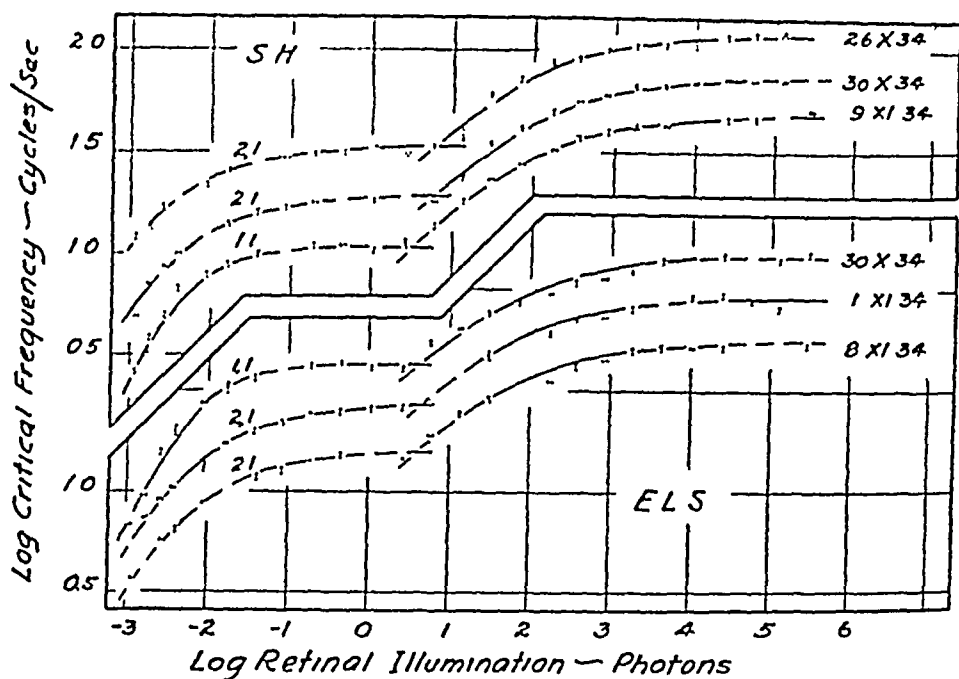


FIG 5 Critical frequency and retinal illumination for a centrally located 19° field. Each individual measurement as taken in the course of a run is shown as a dot. For convenience, the separate runs (dated to the right) have been spaced 0.2 log unit apart on the vertical axis, the values on the ordinate scale refer only to the topmost run for each investigator. The numbers attached to the rod curves indicate the values of m and n in equation (3) used in drawing them.

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data are easily identified with rod activity at low intensities and with cone activity at high intensities

2 The curves describing the rod data are the same for all areas, differing only in the values of the associated dimensional constants which control the location of the curves on the coordinate axes. Similarly, the curves for the cone data are the same for all areas, the tendency for an increase in maximal frequency with area is the expression merely of the value of a constant which determines the position of the data on the frequency axis. Area, therefore, does not influence the fundamental nature of the flicker relation through each receptor system, but merely alters the extraneous constants of the relation.

3 The curves which describe the measurements are represented by two equations, one for rods and one for cones, both equations are derived from the stationary state descriptive of the initial event in the photoreceptor process.

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ISOLATION FROM BEEF PANCREAS OF CRYSTALLINE TRYPSINOGEN, TRYPSIN, A TRYPSIN INHIBITOR, AND AN INHIBITOR TRYPSIN COMPOUND

BY M. KUNITZ AND JOHN H. NORTHROP

*(From the Laboratories of The Rockefeller Institute for Medical Research,
Princeton N. J.)*

(Accepted for publication November 15 1935)

The isolation of trypsin from active pancreatic extract (1) and of chymo trypsinogen and chymo trypsin (2)¹ from fresh inactive pancreas has been described in previous papers. Crystalline trypsinogen has also been obtained (3) from inactive cattle pancreas and transformed into active trypsin which may then be crystallized much more readily than by the earlier method. During the course of this work a polypeptide which has a powerful inhibiting effect on trypsin, as well as a compound of this substance with trypsin, was also obtained in crystalline form (4). The present paper contains detailed descriptions of methods of preparing these substances and a brief description of their properties.

GENERAL PROPERTIES

Trypsinogen

Trypsinogen is obtained as small triangular prisms (Fig. 1). When these crystals are dissolved in neutral solution the trypsinogen is rapidly transformed into active trypsin and it has, therefore, been impossible so far to recrystallize trypsinogen. The original crystallization occurs without activation owing to the presence of the inhibitor and if inhibitor is added to a solution of trypsinogen recrystallization may be carried out without activation. Numerous attempts have been made to recrystallize inhibitor free trypsinogen under conditions

¹ It has recently been found that poorly formed needle shaped protein crystals which have about the same activity as the usual chymo trypsin crystals may be obtained from the mother liquor of the chymo-trypsin crystallization. The properties of these new crystals are now being investigated.

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The transformation of trypsinogen into trypsin is accelerated by the addition of trypsin or enterokinase or concentrated solutions of magnesium sulfate or ammonium sulfate (5). The addition of inhibitor retards activation by all three methods and a large quantity of inhibitor will completely prevent activation. A solution of trypsinogen

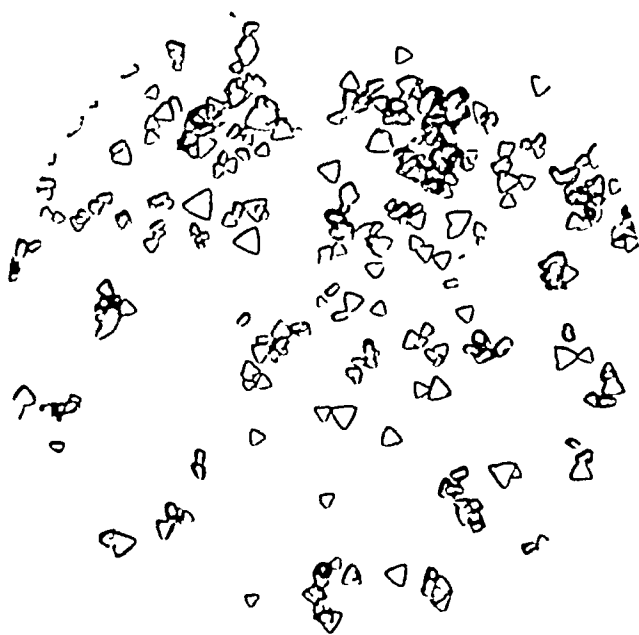


FIG. 1. Trypsinogen crystals.

to which inhibitor has been added behaves, therefore, just as the crude trypsinogen solution previously described (2). The fact that activation is accelerated by the addition of trypsin indicates that activation is autocatalytic and this is borne out by the kinetics of the reaction as shown in Fig. 2. Under these conditions the reaction follows quite closely that of a simple autocatalytic reaction. The rate of activation depends on the pH and is maximum at pH 7.0-8.0. It follows from this that if trypsinogen could be prepared completely free from active trypsin it would remain inactive. Owing to the extremely minute amounts of trypsin required to activate, however,

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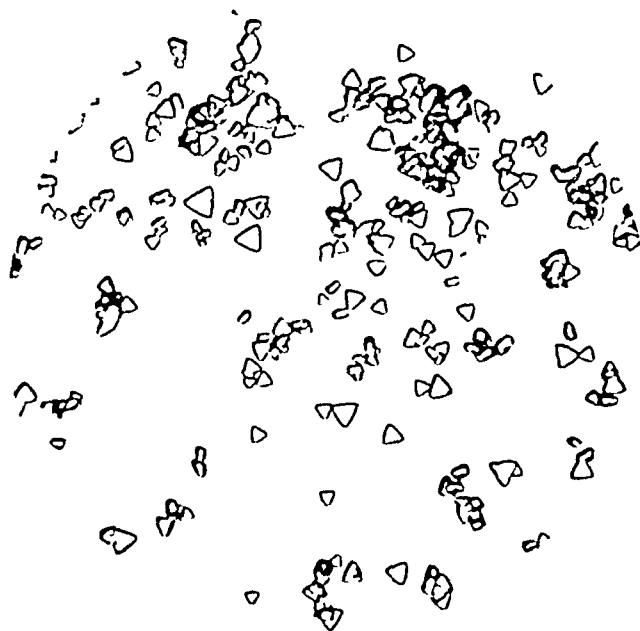


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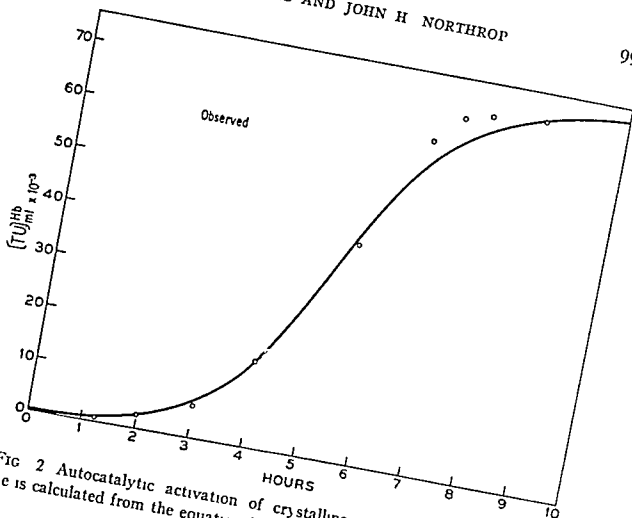


FIG 2 Autocatalytic activation of crystalline trypsinogen The smooth curve is calculated from the equation for a simple autocatalytic reaction

$$KA_0 t = 2.3 \log \frac{A(A - A_0)}{A_0(A - A)}$$

where

$$K = 14.6$$

$$A = 0.072 (T U)_{Hb}$$

$$A_0 = 0.0003 (T U)_{Hb}$$

Crystalline trypsinogen—10 gm crystalline filter cake dissolved in 200 ml N/400 hydrochloric acid 200 ml 5 per cent trichloroacetic acid added Left at 20 C for 1/4 hour Filtered Precipitate dissolved in 25 times its weight of N/50 HCl drochloric acid Ammonium sulfate dissolved in 25 times its weight of N/200 hydrochloric acid Filtered Filter cake dissolved in 3 times its weight of N/200 hydrochloric acid and dialyzed against N/200 hydrochloric acid at 6 C 2 ml dialyzed solution plus 2 ml N/1 pH 5.0 acetate buffer plus 16 ml water at 8 C 1 ml samples removed at intervals and added to 9 ml N/75 hydrochloric acid Activity $\frac{h_1}{h_2}$ the hemoglobin method

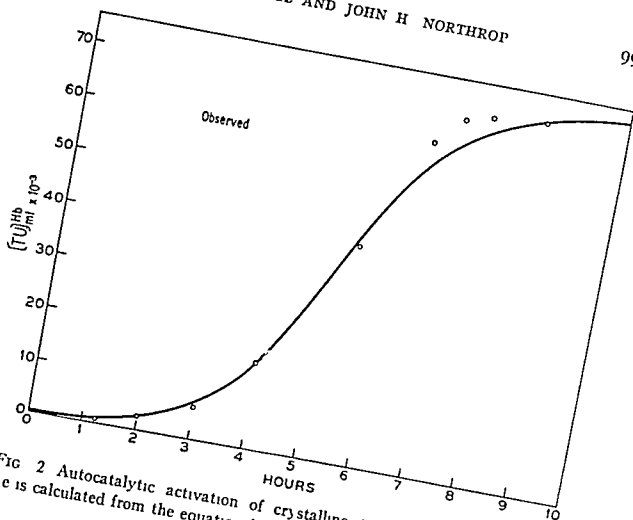


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Trypsin

Crystalline trypsin obtained from inactive pancreatic extract (Fig. 3) has as a rule slightly lower activity than that obtained from

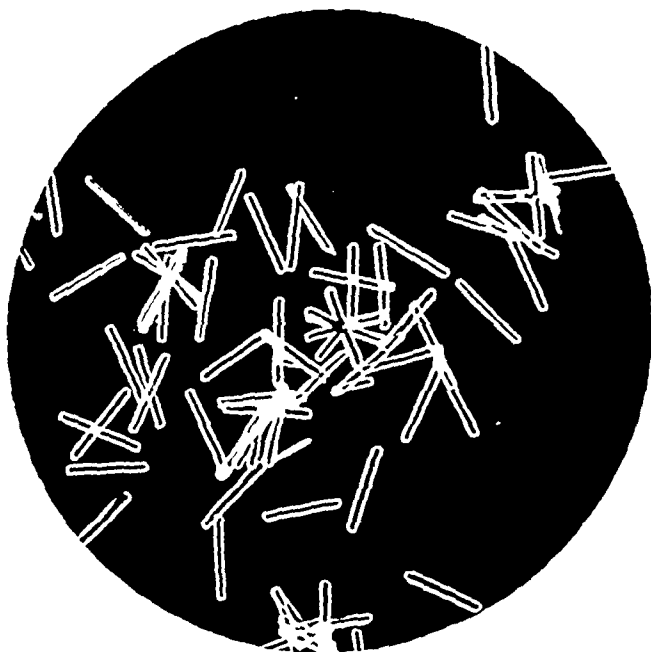


FIG. 3. Crystalline trypsin.

active extracts. This is due partly to the presence of small amounts of inactivated trypsinogen and partly to the presence of some inhibitor. The activity may be increased to the maximum value by repeated crystallization or more rapidly by precipitation with trichloroacetic acid. Either process results in trypsin having the same activity as that prepared from active pancreatic extract (see Table I). The composition, molecular weight, specific activity, etc. on various substrates are the same as previously described for trypsin from active pancreatic extract. These values are summarized in Table II.

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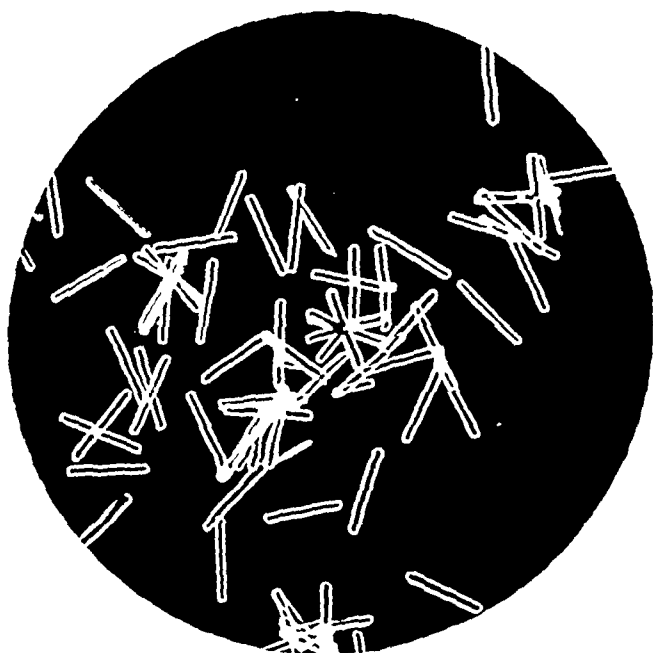


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The trypsin described above appears to be identical with the "proteinase" recently described by Waldschmidt Leitz and Akabori (6). Proteinase was originally described (6) as having a lower activity on clupean than crystalline trypsin but according to Holter and the writers (7) both enzymes hydrolyze clupean at the same rate and to the same extent. This result has also been recently obtained by Professor Waldschmidt Leitz (personal communication from Professor Waldschmidt Leitz).

TABLE I
Activity of Various Preparations of Crystalline Trypsin

Preparation	IT U _{mg} \ determined by		
	Gelatin viscosity pH 4.0	Hemo- globin	Casein mmol
No. 50 prepared from crystalline trypsinogen—1 × crystallized	71	0.10	0.15
3 ×	73	0.12	0.15
5 ×	75	0.12	0.14
8 ×	90	0.14	0.15
3 × crystallized precipitated + trichloroacetic acid (See Par. 1-5)		0.16	
Crystals prepared from active pancreatic juice—1 × crystallized	98	0.11	
3 ×	120	0.15	
5 ×	90	0.16	0.16
	100		

Inhibitor

It has long been known that pancreatic extracts contain some substance which inhibits trypsin and this was clearly brought out by Willstätter and Rhodewald (8). It has been suggested by Dyckerhoff, Miehler, and Tadsen (9) that trypsin exists in fresh pancreas as a compound with the inhibitor so that the activation of this inactive compound consists merely in the removal of the inhibitor and that no special inactive form of trypsin, i.e., trypsinogen, exists. The actual isolation of trypsinogen, as described in the present paper, shows that this explanation is incomplete. The inhibitor, however, does play a very important part in regulating the activation of trypsinogen and there is no doubt that in partly activated pancreatic extracts more or less active trypsin occurs in the form of an inactive comp-

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TABLE II
Analysis and General Properties

	Tryp mg/gm	Trypsin	Inhibitor trypsin compound		Inhibitor
			Natural	Synthetic	
Elementary analysis per cent dry weight, calcd	(C 50.1 H 6.9 N 15.3 S 1.1 P —)	(50.2 6.6 16.13 1.1 —)	(18.0 6.9 15.4 — —)	(— — — — —)	(38.6 7.6 11.25 — —)
Found ash free*					
Protein nitrogen is per cent of total nitrogen		93	76	73	0
Amino nitrogen is per cent total nitrogen formol					6.5
Van Slyke					6.5
Amino nitrogen is per cent total nitrogen after acid hydrolysis					80-90
Tyrosine + tryptophane milliequivalents tyrosine per mg total nitrogen		4.3×10^{-3}	3.7×10^{-3}	3.5×10^{-3}	2.1×10^{-3}
Optical rotation $[\alpha]_D^{25}$ per mg nitrogen		-0.26°	-0.35°	-0.53°	-0.65°
		w/10 pH 5.5 acetate			In distilled water
Molecular weight by osmotic pressure in 0.25 saturated magnesium sulfate 6°C		36,500	40,000		6,000
Substrate					
Hemoglobin		0.17			
Casein S	—	2.4			
Specific activity [I U] per mg protein nitrogen†		0.16			
Casein I	—	100			
Gelatin V	—	<0.1			
Rennet	—	1500			
Clot blood	—	0.6			
Sturin I	—	0.8			
Clupein I	—	+			
Total digestion casein pH 8.0 ml w/50 sodium hydroxide per 5 ml 5 per cent casein		9-11			

* The trypsinogen and trypsin were prepared for analysis by precipitation with trichloroacetic acid followed by washing with trichloroacetic acid, distilled water, and alcohol and then dried with ether. The crystals of inhibitor-trypsin compound were washed with distilled water and alcohol and dried with ether. The inhibitor was dried in the form of filter cake with saturated magnesium sulfate. The analytical results are therefore of somewhat uncertain significance since they were not obtained upon the unchanged active preparations.

The elementary analyses were carried out by Dr. Elek in Dr. P. A. Levene's laboratory.

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Elementary analysis per cent dry weight, calculated ash free*	C	50.1	50.2	48.0		38.6
	H	6.9	6.6	6.9		7.6
	N	15.3	16.13	15.4		11.25
	S	1.1	1.1			
	P	—	—	—	—	—
Protein nitrogen is per cent of total nitrogen			93	76	73	0
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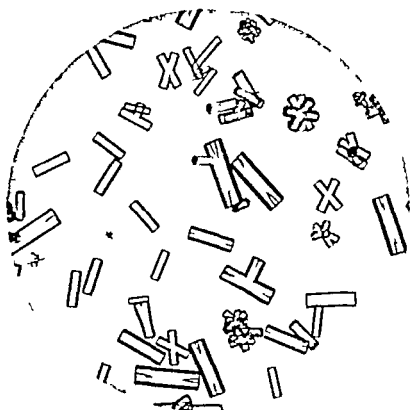


FIG 4 Inhibitor crystals

low but after acid hydrolysis amounts to 80-90 per cent of the total nitrogen so there is reason to believe that the substance is made up largely of amino acids

Reaction between Inhibitor and Trypsin

When a solution of the inhibitor is mixed with a solution of trypsin of equal molecular strength at pH 7.0 the activity of the mixture decreases rapidly with time and after about $\frac{1}{2}$ hour at 6°C it is completely inactive as measured by the digestion of hemoglobin If the

inhibitor The inhibitor (Fig 4) has the general properties of a polypeptide It gives a faint biuret test and is precipitated by saturated magnesium sulfate or 0.7 saturated ammonium sulfate, but is not precipitated nor changed by 2.5 per cent trichloroacetic acid, either hot or cold nor by boiling It diffuses slowly through a celloidion membrane and has a molecular weight, by osmotic pressure of about 6,000 The carbon and nitrogen content (Table II) appear to be lower than usual for proteins The amino nitrogen content is

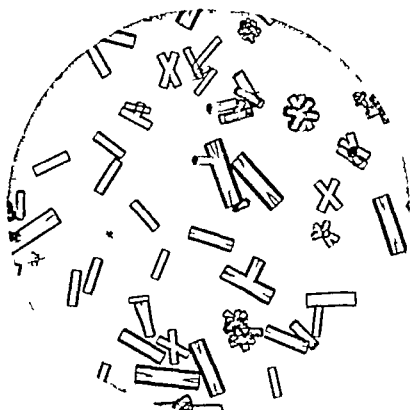


FIG 4 Inhibitor crystals

low but after acid hydrolysis amounts to 80–90 per cent of the total nitrogen so there is reason to believe that the substance is made up largely of amino acids

Reaction between Inhibitor and Trypsin

When a solution of the inhibitor is mixed with a solution of trypsin of equal molecular strength at pH 7.0 the activity of the mixture decreases rapidly with time and after about $\frac{1}{2}$ hour at 6°C it is completely inactive as measured by the digestion of hemoglobin If the

solution is allowed to stand in the pH range of 7.0-3.0 it remains inactive upon addition to hemoglobin digestive mixture (10), but if titrated to pH 10 before addition to hemoglobin the activity rapidly reappears and in about $\frac{1}{2}$ hour will have completely returned. The cycle may be repeated indefinitely. The inhibitor evidently reacts with trypsin to form an addition compound which dissociates in acid

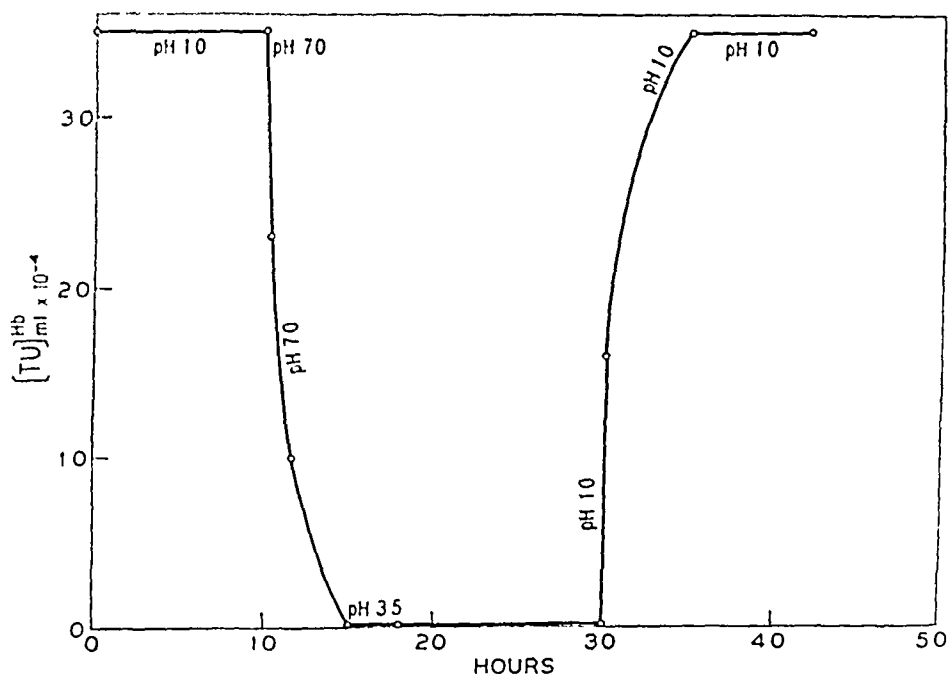


FIG. 5 Effect of standing at 6°C and various pH on the activity of inhibitor-trypsin compound

Solution of inhibitor-trypsin compound pH 5.0 1.3 mg P N/ml diluted 1/500 with 0.0025 N hydrochloric acid pH changed as indicated. 1 ml samples taken at various time intervals, added immediately to standard hemoglobin solution, and the amount of digestion determined after 10 minutes

solution. Both dissociation and combination require measurable time intervals so that the reaction does not appear to be ionic. An experiment illustrating this inactivation and reactivation is shown in Fig. 5. This inactivating effect is also apparent when the activity is measured by the digestion of casein, clotting of blood, digestion of

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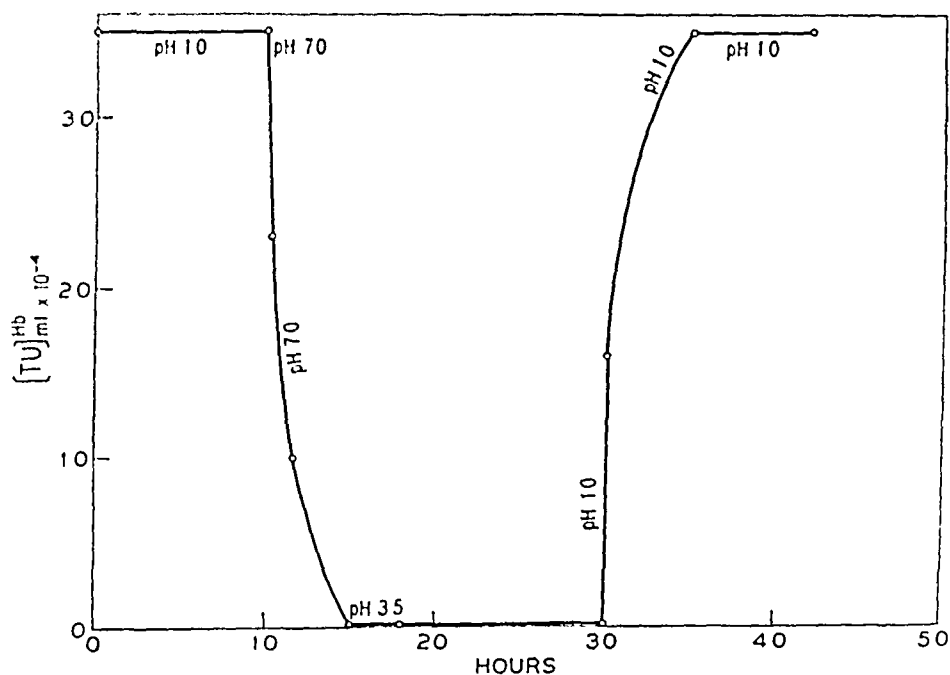


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sturin, or by the activation of chymo trypsinogen or trypsinogen in the presence of salt. The substance also inhibits chymo trypsin but to a less marked extent.

Inhibitor Trypsin Compound

This substance is obtained in the form of hexagonal many faced crystals (Fig. 6). It consists of one molecule of inhibitor combined with one molecule of trypsin (Table III). These may be separated



FIG. 6. Crystals of inhibitor trypsin compound.

by precipitation with trichloroacetic acid which precipitates the trypsin and leaves the inhibitor in solution. As described under "inhibitor," the compound when dissolved in acid solution and added to protein solutions possesses the full tryptic activity but if allowed to stand for a short time at pH 7.0 and then added to the protein solution it is completely inactive. It differs in one marked respect from trypsin in that it is not adsorbed by egg albumin under the conditions described by Waldschmidt-Leitz (11), whereas trypsin itself is adsorbed under these conditions. The molecular weight by osmotic pressure is 40,000.

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TABLE III
Composition of Inhibitor Trypsin Compound (Corrected for Ash)

	Trypsin	Inhibitor	Inhibitor trypsin compound	
	Observed	Observed	Observed	Calculated assuming 1 molecule trypsin (36,000) + 1 molecule inhibitor (6,000)
C	50.2	38.6	48.0	48.6
H	6.60	7.62	6.90	6.70
N	16.15	11.25	15.40	15.40
O	27.1	12.50	29.70	29.50

Molecular weights		
Observed	Observed	Observed
56,700 \pm 2,000	6,500 \pm 1,000	40,000
	Calculated* from per cent composition of trypsin inhibitor and inhibitor trypsin compound assuming molecular weight of trypsin 56,500	Calculated from molecular weight trypsin \pm inhibitor
		42,500
	X	
	C	8,500
	H	(15,000)
	N	6,400
	O	7,400

* X = gm inhibitor in 1 gm mol inhibitor-trypsin compound

C_c = per cent carbon in compound

C_t = " " trypsin

C_i = " " inhibitor

$$\text{Then } X = \frac{36,500}{100} \frac{C_t - C_c}{C_c - C_i}$$

X is calculated from the percentages of the other elements in the same way as shown above for carbon

EXPERIMENTAL METHODS

The methods of preparing these compounds are given in the following sections. The yields reported represent average figures and may vary considerably in individual preparations but the proportion of precipitate to solvent specified in the text must be accurately adhered to. Weights of precipitates refer to the weight of the filter cake that is removed from the Buchner funnel. It is essential

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		Calculated* from per cent composition of trypsin inhibitor and inhibitor trypsin compound is summing molecular weight of trypsin 36,500		Calculated from molecular weight trypsin + inhibitor	
		X		42,500	
		C 8,500			
		H (15,000)			
		N 6,400			
		O 7,400			

* X = gm inhibitor in 1 gm mol inhibitor-trypsin compound

C_c = per cent carbon in compound

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for the success of the preparation that these filtrations be as complete as possible. Large Büchner funnels must be used, the filter cake pressed with a spatula so as to fill all the cracks and the filtration continued until little or no foam is drawn through the funnel. As a rule the dry precipitate should form a layer not more than 2-3 mm thick. The preparations are all quite stable in the form of a moist filter cake if kept in the ice box. Permanent dry preparations may be obtained by allowing the filter cake from saturated magnesium sulfate to stand near the coils of a mechanical refrigerator. Under these conditions the water evaporates and a mixture of the dry crystals with the anhydrous salt remains. Such dry preparations keep indefinitely.

pH determinations were made on a test plate by mixing 1 drop of the Clark indicator with 1 drop of the solution. Standards were prepared by mixing 1 drop of the indicator with 1 drop of the standard buffer. This method of course gives only apparent pH values which may be considerably removed from the true pH of the solution. The method, however, is perfectly adequate for reproducing the necessary conditions.

The saturated magnesium sulfate and ammonium sulfate solutions were prepared at 20°C.

The method of preparation varies somewhat depending upon the desired product. The first method describes a complete fractionation whereby all of the substances may be obtained starting from fresh inactive cattle pancreas. This is the best method for obtaining chymo trypsinogen and fair yields of trypsinogen but does not always give good yields of inhibitor trypsin compound. The second method is the most convenient and reproducible method for obtaining trypsin and inhibitor trypsin compound but yields no trypsinogen. The third method is the most efficient for the preparation of inactive trypsinogen. The fourth method describes the conversion of trypsinogen into active trypsin and the fifth method describes the isolation and crystallization of trypsin from active pancreas.

I Isolation of Crystalline Chymo Trypsinogen, Trypsinogen, and Trypsin Inhibitor from Fresh Cattle Pancreas

1 Preliminary Purification and Concentration²

Remove pancreas from cattle immediately after slaughter and immerse at once in enough ice cold 0.25 N sulfuric acid to cover the glands. Remove fat and connective tissue and mince in a meat chopper within a few hours. Suspend 3 liters

² The method is the same as that already described for the preparation of chymo trypsinogen (2) up to the crystallization of this compound.

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of minced pancreas in 6 liters of 0.25 N sulfuric acid at 5°C and allow suspension to stand at about 5°C for 18–24 hours. Strain the suspension through gauze, resuspend the residue in an equal volume of cold 0.25 N sulfuric acid, and strain through gauze immediately. Reject residue. Dissolve 242 gm of solid ammonium sulfate in each liter of combined filtrate and washings. Filter through fluted paper (S and S No. 1450 $\frac{1}{2}$) in cold room. Reject precipitate. Dissolve 205 gm of solid ammonium sulfate in each liter of filtrate. Heavy precipitate forms. Remove foam and allow to settle for 2 days at 5°C. Decant supernatant solution and filter residue with suction through hardened paper (S and S No. 575) on a large funnel. Yield about 100 gm precipitate. Reject filtrate. Dissolve each 100 gm precipitate in 300 ml water, add 200 ml saturated ammonium sulfate, stir in 5 gm Standard Super Cel (Celite Corporation)³ and filter with suction through soft paper (S and S No. 1450 $\frac{1}{2}$). Reject precipitate. Add slowly 205 gm solid ammonium sulfate to each liter of filtrate and filter with suction through hardened paper (S and S No. 575). *Precipitate A* about 90 gm (mixture of crude chymo-trypsinogen, trypsinogen, and inhibitor). Reject filtrate.

2 Crystallization of Chymo-Trypsinogen

Dissolve each 90 gm of precipitate A in 135 ml water, add 45 ml saturated ammonium sulfate, then adjust to pH 5.0 (brick red color with 0.01 per cent methyl red solution on test plate) by addition drop by drop of about 2 ml 5 N sodium hydroxide. Allow to stand for 2 days at 20–25°C. A heavy crop of chymo-trypsinogen crystals gradually forms. Filter with suction through hardened paper (*Filtrate Tg*). Wash crystalline filter cake with 0.25 saturated ammonium sulfate and finally with saturated ammonium sulfate, and store at 5°C. Yield about 25 gm. For recrystallization and activation see (2).

3 Crystallization of Trypsinogen

Adjust filtrate and washings from chymo-trypsinogen crystallization (*Filtrate Tg*) to pH 3.0 (pink with 0.01 per cent methyl orange on test plate) with about 1 ml 5 N sulfuric acid per 100 ml filtrate. Dissolve 30.4 gm of solid ammonium sulfate in each 100 ml of filtrate and filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (40 gm) in 120 ml water, add 80 ml saturated ammonium sulfate and 2 gm filter cell, and filter with suction through soft paper. Wash paper with 0.4 saturated ammonium sulfate. Reject precipitate. Add slowly 100 ml saturated ammonium sulfate to each 100 ml of combined filtrate and washings. Remove foam and filter with suction through hardened paper, size 18.5 cm or larger. Reject filtrate. Wash precipitate on funnel with saturated magnesium sulfate in 0.02 N sulfuric acid to remove excess of ammonium sulfate. The washing with saturated magnesium sulfate must be done rapidly, otherwise the precipitate is partly dissolved. Saturated magnesium sulfate is

³ This useful material was suggested by Dr. M. L. Anson.

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poured on the precipitate to a height of about 5 mm allowed to filter for a few minutes then the excess of saturated magnesium sulfate is decanted, and filtration is continued until complete. Dissolve precipitate (30 gm) in 30 ml 0.4 M borate buffer pH 9.0 at 2–5°C (in an ice water bath) add more borate buffer drop by drop to pH 8.0, measure solution, and add equal volume of saturated magnesium sulfate, mix and allow solution to stand in ice box at about 5°C (*Solution B*). Short triangular prisms of trypsinogen appear in the course of 2–3 days. If the solution is inoculated with crystals of trypsinogen crystallization is much more rapid but the crystals are not so well formed. (If crystallization is delayed more than 4–5 days, or if the material has become partly active during the preparation crystals of trypsin may appear.)

Filter the crystals with suction at 5°C (*Filtrate C*). The precipitate (about 10 gm) is washed on the funnel several times with cold 0.5 saturated magnesium sulfate made up in 0.1 M borate buffer pH 8.0 and finally with saturated magnesium sulfate made up in 0.1 N sulfuric acid at room temperature. The crystals are then dried in an electric refrigerator at 5°C and stored in the ice box. The dried material generally contains about 40 per cent of trypsinogen protein and 60 per cent magnesium sulfate. For further purification of trypsinogen by means of trichloroacetic acid see legend to Fig. 2. For activation into trypsin see Section IV.

4 Crystallization of Inhibitor Trypsin Compound

Combine Filtrates C and washings from several trypsinogen crystallizations (1000 ml). Adjust to pH 3.0 with 5 N sulfuric acid and saturate with magnesium sulfate by stirring for 15 minutes at 25°C with an excess of crystals of magnesium sulfate. Filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (150 gm) in 750 ml water and add 750 ml 5 per cent trichloroacetic acid. Heat the mixture at 80°C for 5 minutes cool to 25°C and filter with suction through hardened paper. Reject precipitate. Adjust filtrate to pH 3.0 with 5 M sodium hydroxide (about 3 ml per 100 ml of solution). Saturate with crystals of magnesium sulfate. Filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (15 gm) in 45 ml 0.02 M hydrochloric acid, add 1.5 gm of crystalline trypsin allow to stand until the trypsin is dissolved, adjust to pH 8.0 by addition of 0.4 M borate pH 9.0. Allow to stand for 1 hour at 5°C. Adjust to pH 5.5 by addition of 5 N sulfuric acid saturate with crystals of magnesium sulfate at 25°C, allow to stand for 2 days at 20–25°C. Hexagonal crystals of inhibitor trypsin compound gradually appear mixed with amorphous precipitate. Filter with suction. Wash precipitate on paper with 0.5 saturated magnesium sulfate. This dissolves the amorphous precipitate. Residue on paper—crystals of inhibitor trypsin compound. Yield about 5 gm. Filtrate and washings, when saturated with crystals of magnesium sulfate on standing may yield more crystals of inhibitor trypsin compound.

Recrystallization of the Inhibitor Trypsin Compound—Dissolve the filter cake

poured on the precipitate to a height of about 5 mm allowed to filter for a few minutes then the excess of saturated magnesium sulfate is decanted, and filtration is continued until complete. Dissolve precipitate (30 gm) in 30 ml 0.4 M borate buffer pH 9.0 at 2–5°C (in an ice water bath) add more borate buffer drop by drop to pH 8.0, measure solution, and add equal volume of saturated magnesium sulfate, mix and allow solution to stand in ice box at about 5°C (*Solution B*). Short triangular prisms of trypsinogen appear in the course of 2–3 days. If the solution is inoculated with crystals of trypsinogen crystallization is much more rapid but the crystals are not so well formed. (If crystallization is delayed more than 4–5 days, or if the material has become partly active during the preparation crystals of trypsin may appear.)

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Recrystallization of the Inhibitor Trypsin Compound—Dissolve the filter cake

of crystals (5 gm) in 50 ml $\pi/10$ acetate buffer pH 5.5, filter through Whatman No. 12 fluted paper. Saturate with crystals of magnesium sulfate and allow to stand 1 day at 20–25°C. Hexagonal crystals of the inhibitor-trypsin compound rapidly appear. Yield about 3 gm filter cake.

5 *Isolation of Crystalline Trypsin Inhibitor and of Crystalline Trypsin from a Solution of Crystalline Inhibitor-Trypsin Compound*

Dissolve 1 gm crystalline filter cake (of 3 times recrystallized inhibitor-trypsin compound) in 10 ml water and add 10 ml 5 per cent trichloroacetic acid, allow to stand at 20°C for 30 minutes until precipitation is about complete. Filter with suction (*Filtrate In*). The precipitate (*Ts*) is worked up for trypsin, as described below.

(a) *Crystallization of Trypsin Inhibitor*—Heat the trichloroacetic acid filtrate (*In*) for 5 minutes at 80°C, cool, and filter through fluted Whatman No. 42 paper. Reject precipitate. Adjust filtrate to pH 3.0 with 5 π sodium hydroxide. Dissolve 5.6 gm of solid ammonium sulfate in every 10 ml of filtrate. Filter with suction. Reject filtrate. Dissolve precipitate (0.25 gm) in 2.5 ml water, adjust to pH 5.5 with 0.4 π borate pH 9.0. Add equal volume of saturated ammonium sulfate. Filter through No. 42 filter paper. Wash paper with 0.5 saturated ammonium sulfate. Add more saturated ammonium sulfate to filtrate and washings combined until slight precipitate forms. The amorphous precipitate gradually changes into long hexagonal prisms. Allow to stand for 2 days at 20°C. Filter with suction. Filter cake 0.15 gm of inhibitor crystals. Wash filter cake with saturated magnesium sulfate if it is desired to have the crystals free from ammonium salt, and recrystallize with magnesium sulfate.

Recrystallization—Dissolve crystals (0.15 gm) in 1.5 ml of $\pi/10$ acetate buffer pH 5.5. Add 7.5 ml saturated ammonium sulfate (or 7.5 ml saturated magnesium sulfate plus a few crystals of solid magnesium sulfate). Allow to stand at 20°C for 1 day. Crystals of inhibitor gradually appear. Yield about 0.1 gm filter cake.

(b) *Crystallization of Trypsin*—Wash the trichloroacetic acid precipitate (*Ts*) on the filter paper with water to remove the free acid. Dissolve precipitate (0.7 gm) in 20 ml 0.02 π hydrochloric acid. Allow to stand 30 minutes at 20°C. Add 5 gm solid ammonium sulfate. Filter through fluted Whatman No. 42 paper until clear. Dissolve 5 gm of solid ammonium sulfate in the filtrate and filter with suction through 5 $\frac{1}{2}$ cm hardened paper. Reject filtrate. Wash precipitate on paper with saturated magnesium sulfate in 0.02 N sulfuric acid. Dissolve precipitate (0.5 gm) in 0.25 ml water. Cool to 5°C, add about 0.5 ml borate buffer pH 9.0 until the solution reaches pH 8.0 (pink to 0.01 per cent phenol red but not to 0.01 per cent cresol red on test plate). Add 0.5 ml saturated magnesium sulfate. Allow to stand at 5°C. Square prismatic crystals of trypsin rapidly appear. Filter with suction. Yield about 0.25 gm.

of crystals (5 gm) in 50 ml $\pi/10$ acetate buffer pH 5.5, filter through Whatman No. 12 fluted paper. Saturate with crystals of magnesium sulfate and allow to stand 1 day at 20–25°C. Hexagonal crystals of the inhibitor-trypsin compound rapidly appear. Yield about 3 gm filter cake.

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II Isolation of Crystalline Trypsin and Inhibitor Trypsin Compound from Chymo Trypsinogen Free Activated Pancreatic Extract

Preliminary purification and concentration is the same as described under I for preparation of Solution B (Section I 3)

1 Crystallization of Trypsin

Inoculate Solution B (about 100 ml) with trypsin crystals, allow to stand at 5 C for several days. Precipitate of very small crystals of trypsin gradually forms. Occasionally a few triangular trypsinogen crystals may also appear. Filter with suction at 5 C. *Filtrate E*. Filter cake is washed on paper several times with 0.5 saturated magnesium sulfate at 5 C and finally with saturated magnesium sulfate in 0.1 N sulfuric acid at room temperature. Yield 8 gm of filter cake.

2 Recrystallization of Trypsin

Dissolve filter cake (8 gm) in 6 ml 0.02 N sulfuric acid. Add a few drops of 5 N sulfuric acid if solution is incomplete. Cool to 5 C, add 12 ml saturated magnesium sulfate and 6 ml 0.4 M borate pH 9.0. Adjust to pH 8.0 with saturated potassium bicarbonate or 5 N sulfuric acid if necessary. Inoculate. Allow to stand for 1 day at 5 C. Yield 3 gm filter cake.

3 Purification of Trypsin by Trichloroacetic Acid

When first crystallized trypsin sometimes has a slightly low specific activity due partly to the presence of some inhibitor or trypsinogen. The activity may be raised to the maximum value by repeated recrystallization or, more easily, by precipitation with trichloroacetic acid followed by crystallization. The procedure for the latter method is the same as that described under Section I 5 for the preparation of trypsin from inhibitor trypsin compound except that the starting material is trypsin instead of inhibitor trypsin compound.

4 Crystallization of Inhibitor Trypsin Compound

Adjust *Filtrate E* (Section II, 1) to pH 3.0 with 5 N sulfuric acid and saturate with crystals of magnesium sulfate at 25 C. Filter with suction through hardened paper. Reject filtrate. Dissolve precipitate (10 gm) in 50 ml M/16 hydrochloric acid and pour solution with stirring into a large beaker containing 250 ml M/16 hydrochloric acid at 90 C. Cool after 1 minute in running cold water to 25 C (use coils described elsewhere (1) if large quantities of solutions are used). Dissolve 24.2 gm solid ammonium sulfate in each 100 ml of solution, filter through fluted paper. Dissolve 20.5 gm of solid ammonium sulfate in each 100 ml of filtrate. Refilter with suction. Dissolve filter cake (3 gm) in 12 ml water. Cool in ice water, add about 3 ml 0.4 M borate pH 9.0 in order to bring the solution to pH